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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	0010-1108-0 CONT
First Inventor or Application Identifier	Yoko ASAKURA
Title	METHOD OF CONSTRUCTING AMINO ACID PRODUCING BACTERIAL STRAINS, AND METHOD OF PREPARING AMINO ACIDS BY FERMENTATION WITH THE CONSTRUCTED AMINO ACID PRODUCING BACTERIAL STRAINS

JC714 U.S. PTO 09/577005 05/25/00

APPLICATION ELEMENTS
See MPEP chapter 600 concerning utility patent application contents

ADDRESS TO: Assistant Commissioner for Patents
Box Patent Application
Washington, DC 20231

1. ■ Fee Transmittal Form (e.g. PTO/SB/17)
(Submit an original and a duplicate for fee processing)
2. ■ Specification — Total Pages 93
3. ■ Drawing(s) *(35 U.S.C. 113)* — Total Sheets 3
4. ■ Oath or Declaration — Total Pages 6
   a. ■ Newly executed (original or copy)
   b. ☐ Copy from a prior application *(37 C.F.R. §1.63(d))*
   (for continuation/divisional with box 15 completed)
      i. ☐ DELETION OF INVENTOR(S)
      Signed statement attached deleting inventor(s) named in the prior application, see *37 C.F.R. §1.63(d)(2) and 1.33(b).*
5. ■ Incorporation By Reference The entire disclosure of the prior application, is considered to be part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

ACCOMPANYING APPLICATION PARTS

6. ■ Assignment Papers (cover sheet & document(s))
7. ☐ 37 C.F.R. §3.73(b) Statement *(when there is an assignee)* ☐ Power of Attorney
8. ☐ English Translation Document *(if applicable)*
9. ■ Information Disclosure Statement (IDS)/PTO-1449 ■ Copies of IDS Citations (5)
10. ■ Preliminary Amendment
11. ■ White Advance Serial No. Postcard
12. ☐ Small Entity Statement(s) ☐ Statement filed in prior application. Status still proper and desired.
13. ■ Certified Copy of Priority Document(s) (2)
(if foreign priority is claimed)
14. ■ Other: REQUEST FOR PRIORITY; INTERNATIONAL SEARCH REPORT
SEQUENCE LISTING (HARD COPY)

15. If a CONTINUING APPLICATION, *check appropriate box, and supply the requisite information below:*

■ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior International Application No.: PCT/JP99/05175

Prior application information: Examiner: Group Art Unit:

16. Amend the specification by inserting before the first line the sentence:

■ This application is a ■ Continuation ☐ Division ☐ Continuation-in-part (CIP) of International Application No. PCT/JP99/05175 Filed on September 22, 1999, pending

☐ This application claims priority of provisional application Serial No. Filed

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0010-1108-0 CONT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF: :

Yoko ASAKURA et al : NEW APPLICATION

SERIAL NO: NEW APPLICATION :

FILED: HEREWITH :

FOR: METHOD OF CONSTRUCTING AMINO ACID PRODUCING BACTERIAL STRAINS, AND METHOD OF PREPARING AMINO ACIDS BY FERMENTATION WITH THE CONSTRUCTED AMINO ACID PRODUCING BACTERIAL STRAINS

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Prior to examination on the merits, please amend this application as follows.

IN THE SPECIFICATION

Page 1, at the top of the page, delete and insert therefor:

- -TITLE OF THE INVENTION

BACKGROUND OF THE INVENTION

Field of the Invention- -;

line 7, delete

"Background of the Invention";

after line 10, insert:

- -Description of the Background- -.

Page 7, line 6, delete

"Disclosure of the Invention" and insert therefor:

- -SUMMARY OF THE INVENTION- -;

after line 24, insert:

- -DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT- -.

IN THE CLAIMS

--12. (Amended) A glutamic acid-synthesizing gene having a promoter according to [any one of Claims 4 to 8] Claim 4.- -

- -16. (Amended) A method of producing an amino acid or nucleic acid by [the steps of] culturing a coryneform bacterium constructed by the method of [any one of Claims 1 to 11] Claim 1, and having an improved amino acid - or nucleic acid - productivity[, or the coryneform bacterium of Claims 14 or 15] in a culture medium to form and thereby [to] accumulate the [intended] amino acid or nucleic acid in the culture medium, and collecting [it] the amino acid or nucleic acid from the culture medium.- -

- -17. (Amended) A method of producing L-glutamic acid by fermentation, which comprises [the steps of] culturing a coryneform L-glutamic acid-producing bacterium resistant to 4-fluoroglutamic acid in a liquid culture medium to produce and thereby [to] accumulate L-glutamic acid in the culture medium, and collecting [it] the L-glutamic acid from the culture medium.- -

Please add new Claims 18 and 19 as follows:

- -18. A method of producing an amino acid or nucleic acid by fermentation, which comprises culturing the coryneform bacterium of Claim 14, in a culture medium to form and thereby accumulate the amino acid or nucleic acid in the culture medium and collecting the amino acid or nucleic acid from the culture medium.

19. A method of producing an amino acid or nucleic acid by fermentation, which comprises culturing the coryneform bacterium of Claim 15, in a culture medium to form and thereby accumulate the amino acid or nucleic acid in the culture medium and collecting the amino acid or nucleic acid from the culture medium.- -

REMARKS

Claims 12, 16 and 17 have been amended. New Claims 18 and 19 have been added. Hence, Claims 1-19 are now active and under consideration in this case.

Applicants have amended the Claims to clarify the present invention. The specification has been amended to add appropriate section headings. All of the above amendments are supported by the Claims and disclosure as originally filed. No new matter has been added.

Accordingly, it is believed that this application is now in condition for examination on the merits. Favorable consideration is earnestly solicited.

Respectfully submitted,

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SPECIFICATION

Method of constructing amino acid producing bacterial strains, and method of preparing amino acids by fermentation with the constructed amino acid producing bacterial strains

Background of the Invention

The present invention relates to a method of constructing a mutant strain capable of producing amino acids in a high yield, and a method of producing L-amino acids by the fermentation with the mutant.

Methods of constructing mutant strains usable for the production of amino acids by the fermentation can be roughly classified into two methods. One of them comprises introducing random mutations into DNA with a chemical mutagen, and the other comprises the genetic recombination. In the latter method, a strain having an improved capacity of producing an intended substance can be developed by enhancing a gene on a metabolic pathway relating to the biosynthesis of an intended substance, or by weakening a gene of an enzyme relating to the destruction. In this connection, for enhancing an intended gene, a plasmid capable of autonomously replicating independently from the chromosome in a cell has been mainly used.

However, the method of enhancing the intended gene with a plasmid has problems. In particular, the degree of enrichment of the intended gene is variable depending on the number of copies of the plasmid itself. Therefore, for some kinds of intended genes, the copies are often too many in number and, as a result, the expression becomes excessive, the growth is seriously inhibited or the capacity of producing the intended substance is lowered. In such a case, although the degree of the enhancement of the intended gene can be lowered by using a plasmid of a small number of the copies, the variety of the plasmid is limited in many cases, and the intended control of the expression level of the intended gene is impossible.

Another problem is that since the replication of the plasmid is often unstable,

the plasmid is eliminated.

For example, Japanese Patent Unexamined Published Application (hereinafter referred to as "J. P. KOKAI") No:61-268185 discloses a recombinant DNA comprising a DNA fragment containing a glutamate dehydrogenase (GDH)-producing gene (glutamate dehydrogenase gene) derived from a glutamate-producing coryneform bacterium, and a DNA fragment (plasmid) containing a gene necessary for the autonomous replication in the cell. It is also disclosed therein that by introducing the recombinant DNA into a cell, a GDH-enriching strain can be grown to improve the production of substances (such as amino acids and proteins) with microorganisms.

On the other hand, in Japanese Patent No. 2,520,895, the above described recombinant DNA is introduced into Corynebacterium to obtain a strain having the improved enzymatic activity, and L-glutamic acid is produced by the fermentation with the strain. However, the production and yield of L-glutamic acid were yet unsatisfactory. Thus, it is demanded to further improve the productivity of L-glutamic acid. It is reported that the demand had been attained by introducing a recombinant DNA comprising two kinds of genes, i.e. a glutamate dehydrogenase-producing gene derived from a glutamate-producing coryneform bacterium, and an isocitrate dehydrogenase (ICDH)gene, into a glutamate-producing coryneform bacterium.

Further, JP Kokai No.6-502548 discloses an expression system and a secretion system of Corynebacterium comprising a Corynebacterium strain and a secretory cassette comprising the first functional DNA sequence for the expression in the strain, the second DNA sequence encoding for amino acids, polypeptides and/or proteins and the third DNA sequence inserted between the first DNA sequence and the second DNA sequence, wherein the third DNA sequence encodes the protein element selected from PS1 and PS2 which guarantee the secretion of the amino acids, polypeptides and/or proteins. Specifically, the secretion of polypeptides is disclosed therein and in particular, NTG mutagtenesis was conducted with Corynebacterium and a mutant resistant to 4-fluoroglutamate (4FG) which is an analogue to glutamate

is selected and subjected to the transformation with PCGL141. It is described therein that a strain having an enhanced expression of GDH can be obtained from the analogue resistant bacteria. It is also described therein that a mutation was observed in nucleotide sequence No.251 to No.266 of GDH promoter.

Disclosure of the Invention

The object of the present invention is to provide a method of constructing a mutant capable of suitably enhancing or controlling the expression of an intended gene without using a plasmid and also capable of producing amino acids in a high yield, by gene recombination or mutation.

Another object of the present invention is to provide a promoter for GDH capable of imparting a capability of producing glutamic acid in a high yield to a Corynebacterium strain without seriously increasing the amount of by-produced aspartic acid and alanine.

Still another object of the present invention is to provide a GDH gene having a sequence of the above-described promoter for GDH.

A further object of the present invention is to provide a Corynebacterium strain having the above-described gene and capable of producing l-glutamic acid.

A further object of the present invention is to provide a method of producing amino acids by fermentation wherein amino acid-producing microorganism thus constructed is used.

A further object of the present invention is to provide a fermentation method of producing glutamic acid at a low cost by increasing the yield of glutamic acid by using a glutamic acid-producing coryneform bacterium.

The present invention has been completed on the basis of a finding that the above-described problems can be efficiently solved by variously modifying the promoter of amino acid-biosynthesizing genes on a chromosome to control the amount of the expression of the intended genes. Particularly, the invention has been completed on the basis of a finding that the above-described problem can be

efficiently solved by introducing a specific mutation into -35 region or -10 region which is a specific region of the promoter.

Namely, the present invention provides a method of producing coryneform bacteria having an improved amino acid- or nucleic acid-productivity, which comprises the steps of introducing a mutations in a promoter sequence of amino acid- or nucleic acid-biosynthesizing genes on a chromosome of a coryneform bacteria to make it close to a consensus sequence or introducing a change in a promoter sequence of amino acid- or nucleic acid-biosynthesizing genes on a chromosome of Coryneform bacteria by gene recombination to make it close to a consensus sequence, to obtain mutants of the coryneform amino acid- or nucleic acid-producing microorganism, culturing the mutants, and selecting a mutant capable of producing the intended amino acid or nucleic acid in a large amount.

The present invention also provides a promoter for glutamate dehydrogenase (GDH)-producing gene, which has the sequence of (i) at least one DNA sequence selected from the group consisting of CGGTCA, TTGTCA, TTGACA and TTGCCA in -35 region, (ii) TATAAT sequence or the same TATAAT sequence but in which the base of ATAAT is replaced with another base in -10 region, or (iii) a combination of (i) and (ii), wherein the sequence does not inhibit the promoter function.

The present invention also provides a glutamate dehydrogenase-producing gene having the above-described promoter.

The present invention also provides a coryneform L-glutamate-producing microorganism having the above-described gene.

The present invention also provides a process for producing an amino acid by the fermentation, which comprises the steps of culturing a coryneform bacterium constructed by the above-described method and having an improved amino acid-producing capacity in a medium to form and also to accumulate the intended amino acid in the medium, and collecting the amino acid from the medium.

The present invention also provides a process for producing L-glutamic acid by the fermentation, which comprises the steps of culturing a coryneform L-glutamic

acid-producing microorganism resistant to 4-fluoroglutamic acid in a liquid medium to form and also to accumulate L-glutamic acid in the medium, and collecting L-glutamic acid from the medium.

Brief Description of the Drawings

Fig.1 show a flow of construction of GDH gene having a mutant promoter.
Fig.2 show a flow of construction of CS gene having a mutant promoter.
Fig.3 show a flow of construction of shuttle vector carrying lacZ as a reporter gene.

Best Mode for Carrying out the Invention

The term "coryneform glutamic acid producing microorganism" as used herein includes also bacteria which were classified to be the genus Brevibacterium before but now integrated into the genus Corynebacterium [Int. J. Syst. Bacteriol., 41, 255 (1981)] and also bacteria of the genus Brevibacterium which are very close to those of the genus Corynebacterium. Therefore, the mutants used in the present invention can be derived from the coryneform glutamic acid-producing bacteria of the genus Brevibacterium or Corynebacterium shown below. Bacteria of the genus Corynebacterium and those of the genus Brevibacterium will be collectively referred to as "coryneform bacteria" so far as they do not concern the glutamic acid productivity.

Corynebacterium acetoacidophilum ATCC13870
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium callunae ATCC15991
Corynebacterium glutamicum ATCC13032
Brevibacterium divaricatum ATCC14020
Brevibacterium lactofermentum ATCC13869
Corynebacterium lilium ATCC15990
Brevibacterium flavum ATCC14067
Corynebacterium melassecola ATCC17965

Brevibacterium saccharolyticum	ATCC14066
Brevibacterium immariophilum	ATCC14068
Brevibacterium roseum	ATCC13825
Brevibacterium thiogenitalis	ATCC19240
Microbacterium ammoniaphilum	ATCC15354
Corynebacterium thermoaminogenes	AJ12310(FERM 9246)

The amino acids to be produced are not particularly limited so far as the genes concerning the biosynthesis and promoters thereof have been elucidated. Examples of effective enzymes concerning the biosynthesis include GDH, citrate synthase (CS), isocitrate synthase (ICDH), pyruvate dehydrogenase (PDH) and aconitase (ACO) for glutamic acid fermentation.

Enzymes for lysine fermentation including biosynthesis enzymes such as aspartate kinase (AK), dihydrodipicolinate synthase, dihydrodipicolinate reductase, diaminopimelate dehydrogenase and diaminopimelate decarboxylase are also effective. Lysine eccrisis protein (lysE gene) concerning the membrane eccrisis of lysine is also effective.

Effective enzumes for arginine fermentation include N-acetylglutamate synthase, N-acetylglutamate kinase, N-acetylglutamyl phosphate reductase, acetylornithine aminotransferase, N-acetylornithinase, ornithine carbamyltransferase, argininosuccinate synthase, and arginosuccinase. arginine is formed by the reaction catalyzed by these enzymes. These enzymes are effective. These enzymes are coded by enzymes argA, argB, argC, argD, aegE, argF, argG and argH, relatively.

Effective enzymes for serine fermentation includes 3-phosphoglyceric acid dehydrogenase, phosphoserine trans-amylase, phosphoserine phosphatase and the like.

Effective enzymes for phenylalanine fermentation include bio-synthesizing enzymes such as deoxyarabinohepturonic phosphate synthetase, 3-dehydrokinate synthetase, 3-dehydrokinic acid dehydroratase, shikimic acid dehydrogenase,

shikimic kinase, 5-enol pyrvilshikimic acid–3-phosphate synthetase, chorismic acid synthetic enzyme, chorismate synthetase, chorismate mutase, prephenate dehydroratase, and the like. Sugar metabolic enzymes such as transketorase, transaldolase, phosphoenolpyrvic acid synthetic enzyme are also effective.

Effective enzymes for tryptophan fermentation include enzymes belonging to tryptophan operon, in addition to various enzymes effective in the above-mentioned phenylalanine fermentation and various enzymes effective in the above-mentioned serine fermentation.

Effective enzymes for proline fermentation include γ -glutamylkinase, γ -glutamylcemialdehyde dehydrogenase, pyrroline-5-carboxylate reductase, in addition to various enzymes effective in the above-mentioned glutamic acid fermentation.

Effective enzymes for glutamine fermentation include glutamine synthetase, in addition to various enzymes effective in the above-mentioned glutamic acid fermentation.

In the inosine production, it is considered to be useful to enhance the expression of 5-phosphoribosyl 1-diphosphate synthetase, 5-phosphoribosyl 1-diphosphate aminotransferase, phosphoribosylaminoimidazolecarboxamide formyltransferase and the like.

In the guanosine production, it is considered to be useful to enhance the expression of 5'-inosinic acid dehydrogenase and 5'-xanthylic acid aminase, addition to 5-phospholibosyl 1-diphosphate synthetase, 5-phospholibosyl 1-diphosphate aminotransferase, phosphoribosylaminoimidazolecarboxamide formyltransferase and the like.

In the adenosine production, it is considered to be useful to enhance the expression of adenirosuccinate synthase, in addition to 5-phosphoribosyl 1-diphosphoric acid synthetic enzyme, 5-phosphoribosyl 1-diphosphoric acid aminotransferase, phosphoribosylaminoimidazole-carboxamide formyltransferase and the like.

In the nucleotide production, it is considered to be useful to enhance the

expression of phosphoribosyl transferase, inosine kinase, guanosine kinase and adenosine kinase.

In the present invention, a mutant of a coryneform amino acid-producing bacterium is obtained by, introducing a mutation in a promoter sequence of desired amino acid-biosynthesizing genes on a chromosome of a coryneform amino acid-producing bacterium, such as the above-described promoter sequence for GDH, to make it close to a consensus sequence with a chemical or by introducing the mutation by the genetic recombination to obtain a mutant of the coryneform amino acid-producing microorganism.

The term "consensus sequence" is a sequence which appears most frequently in various promoter sequences. Such consensus sequences include, for example, those of E. coli and Bacillus subtilis. The consensus sequence of E. coli is described in Diane K. Hawley and William R. McClure Nuc. Acid. Res. 11:2237-2255(1983), and that of B. subtilis is described in Charles et al. Mol. Gen. Genet 186:339-346(1982).

The mutation may be caused in either only one promoter sequence such as that for GDH or two or more promoter sequences such as those for GDH, citrate synthase (citrate-synthesizing enzyme) (CS) and isocitrate synthase (isocitrate-synthesizing enzyme) (ICDH).

In the present invention, the mutant thus obtained is cultured to obtain the mutant capable of producing a large amount of an intended amino acid.

It was already elucidated that in the fermentation of glutamic acid, GDH derived from a coryneform glutamate-producing microorganism has its own promoter sequence in upstream region thereof [Sahm et al., Molecular Microbiology (1992), 6, 317-326].

For example, the promoter for GDH of the present invention, GDH gene having the promoter sequence for GDH and L-glutamate-producing Corynebacterium strain having this gene can be obtained by, for example, the following methods:

Namely, the strain is subjected to a mutagenesis treatment such as the

irradiation with UV, X-rays or radiation, or treatment with a mutagen to obtain a strain resistant to 4-fluoroglutamic acid on an agar plate culture medium containing 4-fluoroglutamic acid. Namely, the mutagenized cells are spread on agar plates culture medium containing 4-fluoroglutamic acid in such a concentration that it inhibits the growth of the parent, and the mutant thus grown is separated.

Further, the promoter sequence of GDH genes can be replaced with variously modified sequences by site directed mutagenesis, and the relationship between the respective sequences and GDH activity is examined so as to select the ones having a high L-glutamate-productivity.

It is particularly preferred in the present invention that the DNA sequence in -35 region of the prompter for GDH-producing gene is at least one DNA sequence selected from the group consisting of CGGTCA, TTGTCA, TTGACA and TTGCCA and/or the DNA sequence in -10 region of the promoter is TATAAT, or the bases of ATAAT in TATTAT sequence in -10 region is replaced with another base, while they do not inhibit the promoter function. The reason why the strain in which the bases of ATAAT in TATAAT sequence in -10 region is replaced with another base and the promoter function is not inhibited can be selected is as follows: Because a remarkable increase in the specific activity of GDH was observed by merely replacing the first "C" of CATAAT with "T" in wild type -10 sequence (refer to p6-4 in Table 1), it was considered that such a replacement with another base is possible.

The promoter sequence of GDH gene is described in, for example, the above-described Sahm et al., Molecular Microbiology (1992), 6, 317-326. It is described therein as Seq ID No. 1. The sequence of GDH gene itself is also described in Sahm et al., Molecular Microbiology (1992), 6, 317-326 to be Seq ID No. 1.

Similarly, the mutation can be introduced in the promoter for citrate-synthesizing enzyme (CS) or isocitrate-synthesizing enzyme (ICDH).

Thus, the promoters for GDH are those having at least one DNA sequence in -10 region selected from the group consisting of CGGTCA, TTGTCA, TTGACA and

TTGCCA in -35 region and/or or TATAAT sequence or the TATAAT sequence but in which the base of ATAAT is replaced with another base, wherein they do not inhibit the promoter function. Genes for producing glutamate dehydrogenase, which have the above-described promoter, are also provided.

The promoters for CS are those having TTGACA sequence in -35 region and/or TATAAT sequence in -10 region, which do not inhibit the promoter function. CS genes having the above-described promoter are also provided.

Promoters for ICDH are those having TTGCCA or TTGACA sequence in the first or the second promoter in -35 region and/or TATAAT sequence in the first or the second promoter in -10 region which do not inhibit the function of the promoter. The icd genes having the above-described promoter are also provided.

Promoters for PDH are those having TTGCCA sequence in -35 region and/or TATAAT sequence in -10 region, which do not inhibit the function of the promoter. PDH genes having the above-described promoter are also provided.

The present invention also provides coryneform L-glutamate-producing bacterium having the above-described genes.

The promoters for algininosuccinate synthase are those having at least one DNA sequence selected from the group consisting of TTGCCA, TTGCTA, and TTGTCA in -35 region and/or TATAAT sequence in -10 region, or the base of ATAAT in TATTAT sequence is replaced with another base, which do not inhibit the function of the promoter. Argininosuccinate synthase gene having the above-described promoter are also provided.

The present invention also provides coryneform arginine-producing bacterium having the above-described genes.

Amino acids can be obtained by culturing a coryneform bacterium of the present invention, which produces an amino acid, preferably L-glutamic acid, in a liquid culture medium to form and thereby to accumulate the intended amino acid, preferably L-glutamic acid, and collecting the amino acid from the culture medium.

The liquid culture medium used for cultivating the above-described strain of

the bacterium in the present invention is an ordinary nutrition medium containing carbon sources, nitrogen sources, inorganic salts, growth factors, etc.

The carbon sources include carbohydrates such as glucose, fructose, sucrose, molasses and starch hydrolyzates; alcohols such as ethanol and glycerol; and organic acids such as acetic acid. The nitrogen sources include ammonium sulfate, ammonium nitrate, ammonium chloride, ammonium phosphate, ammonium acetate, ammonia, peptone, meat extract, yeast extract and corn steep liquor. When an auxotrophic mutant is used, the required substances are added to the medium as the reagents or natural substances containing them.

The coryneform bacteria usually produce L-glutamic acid under reduced biotin condition. Therefore, the amount of biotin in the medium is restricted or a substance inhibiting the effect of biotin such as a surfactant or penicillin is added.

The fermentation is preferably conducted by shaking the culture or agitating the culture with aeration while the pH of the culture liquid is kept in the range of 5 to 9 for 2 to 7 days. The pH is preferably controlled with urea, calcium carbonate, gaseous ammonia, ammonia water or the like. The culture temperature is preferably 24 to 37℃.

L-glutamic acid thus produced and accumulated in the culture liquid is collected by an ordinary method such as ion-exchange resin method or crystallization method. Specifrrfically, L-glutamic acid is separated by the adsorption on an anion-exchange resin or by the neutralization crystallization.

According to the present invention, the intended amino acid can be obtained in a high yield by introducing a mutation into a promoter region of amino acid-biosynthesizing genes of a coryneform amino acid-producing bacterium to control the expression of the intended genes. In addition, since any elimination of the intended gene does not occur in the bacteria according to the present invention, contrary to the cases using plasmid, the intended amino acid can be stably obtained in a high yield. Thus, the industrial merit of the invention is great.

The present invention provides various promoters, particularly, promoters for

GDH, capable of imparting a power of producing amino acids, particularly glutamic acid, in a high yield to Corynebacterium strains without increasing the amount of by-produced aspartic acid and alanine.

In the present invention, a coryneform L-glutamate-producing bacterium is mutagenized, a strain in which the mutation introduced in a promoter region of GDH gene and which is resistant to 4-fluoroglutamic acid is collected, and the strain is cultured to obtain glutamic acid in a high yield. Thus, the present invention is industrially very advantageous.

The following Examples will further illustrate the present invention.

Example 1: Production of mutant GDH promoter:

A mutant GDH promoter was prepared by site-directed mutagenesis method as follows:

(1) Preparation of GDH genes having various mutant promoters:

The wild type sequence in -35 region and -10 region of a promoter of GDH gene of a coryneform bacteria is shown in sequence 1. The promoter sequence of wild type has already been reported [Molecular Microbiology (1992), 6, 317-326].

The method of preparing a plasmid carrying GDH gene having a mutant promoter is as follows:

As shown in Fig. 1, a chromosomal gene of a wild type strain of a coryneform bacterium ATCC13869 prepared with "Bacterial Genome DNA purification kit" (Advanced Genetic Technologies Corp.) was used as the template for PCR. The gene amplification was conducted by PCR using upstream and downstream sequences of GDH gene. Both ends were blunt-ended. The product thus obtained was inserted in SmaI site of plasmid pHSG399 (a product of Takara Shuzo Co., Ltd.). Then a replication origin taken from plasmid pSAK4 having the replication origin capable of replicating in a coryneform bacterium was introduced into Sal I site of the plasmid to obtain plasmid pGDH. By this method, GDH genes having each above-described promoter sequence can be obtained by using a primer having each of the

sequence of Seq ID No. 1 to Seq ID No. 6 shown in the Sequence Listing as the upstream primer for GDH gene, respectively. It was confirmed by sequencing the PCR amplified fragment that any mutation, other than the introduced mutation in the promoter sequence, was not occured in the amplified fragment. pSAK4 is constructed as follows: previously obtained plasmid pHK4 (J. P. KOKAI No.5-7491] having an autonomous replication origin derived from plasmid pHM1519 [Agric. Biol. Chem., 48, 2901-1903 (1984)] ,which is capable of autonomously replicating in Corynebacterium microorganism, is digested with restriction enzymes BamHI and KpnI to obtain a DNA fragment having the replication origin. Then the fragment thus obtained is blunt-ended with DNA-Blunting Kit (Blunting kit of Takara Shuzo Co., Ltd.). After the ligation with SalI linker, the product thus obtained was inserted into Sal I site of pHSG299 (a product of Takara Shuzo Co., Ltd.) to obtain plasmid pSAK4.

(2) Comparison of the degrees of expression of GDH having each promoter sequence:

Each plasmid prepared as described above was introduced into wild type strain of coryneform bacterium ATCC13869 by electroporation method (refer to J. P. KOKAI No. 2-207791. For comparing the degrees of expression of GDH for these strains, the specific activity of GDH was determined by the above-described method of Sahm et al. The results are shown in Table 1.

Table 1

Strain	Promoter sequence		Specific activity of GDH	Relative value
	-35	-10		
ATCC13869	TGGTCA	CATAAT	7.7	0.1
/pGDH	TGGTCA	CATAAT	82.7	1.0
/p6-2	CGGTCA	CATAAT	33.1	0.4
/p6-4	TGGTCA	TATAA.T	225.9	2.7

/p6-3	TTGACA	TATAAT	327.2	4.0
/p6-7	TTGCCA	TATAAT	407.0	4.9
/p6-8	TTGTCA	TATAAT	401.3	4.9

ATCC 13869/p6-2 through ATCC 13869p6-8/ corresponded to the sequences of Seq ID No. 2 through Seq ID No.6, respectively. These sequences were the same as the sequence No.1 (wild type) except that the underlined parts were changed as follows:

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Sequence
No. 1  5'-TTAATTCTTTGTGGTCATATCTGCGACACTGC CATAATTTGAACGT-3'
2                    CGGTCA                 CATAAT
3.                   TGGTCA                 TATAAT
4.                   TTGACA                 TATAAT
5.                   TTGCCA                 TATAAT
6.                   TTGTCA                 TATATT
```

These were those of synthetic linear doubled stranded DNA.

Example 2: Preparation of mutant strains:

(1) Preparation of mutant strains resistant to 4-fluoroglutamic acid:

AJ13029 is a mutant strain producing glutamic acid and disclosed in W096/06180. Although it does not produce glutamic acid at a culture temperature of 31.5℃, it produces glutamic acid even in the absence of a biotin-inhibitor when the culture temperature is shifted to 37 ℃. In this Example, Brevibacterium lactofermentum AJl3029 strain was used as the parent strain for preparing the mutant strains. As a matter of course, any of glutamic acid-producing strains other than AJ13029 can be used as a parent strain for preparing mutant strains resistant to 4-fluoroglutamic acid.

AJ13029 were cultured on a CM2B agar medium (Table 2) at 31.5℃ for 24 hours to obtain the bacterial cells. The cells were treated with 250μg/ml aqueous solution of N-methyl-N'-nitro-N-nitrosoguanidine at 30℃ for 30 minutes. Then a suspension of the cells having a survival rate of 1 % was spread on agar plates culture medium (Table 3) containing 4-fluoroglutamic acid (4FG). Colonies were formed after incubating the plate at 31.5℃ for 20 to 30 hours. In this experiment, a slant medium containing 1 mg/ml of 4FG was prepared at first, and then a layer of the same medium without 4FG was formed thereon horizontally. Thus, 4FG concentration gradient was obtained on the surface of the agar medium. When the plate was inoculated with the mutant cells obtained as described, a boundary line was formed at a border of the growing limit of the strain. Bacterial trains which formed colonies in a area containing 4FG of a concentration higher than that of the boundary line were taken. Thus, about 50 strains resistant to 4FG were obtained from about 10,000 mutagenized cells.

Table 2 CM2B agar medium

Ingredient	Concentration
Polypeptone (Nippon Seiyaku Co.)	1.0 %
Yeast extract (Difco Co.)	1.0 %
NaCl	0.5 %
d-Biotin	10 μg/l
Agar	1.5 %
(pH 7.2: adjusted with KOH)	

Table 3 Agar medium

Component	Amount in one liter of water
Glucose	10g
$MgSO_4 \cdot 7H_2O$	1g

$FeSO_4 \cdot 7H_2O$	0.01g
$MnSO_4 \cdot 4\text{-}6H_2O$	0.01 g
Thiamine hydrochloride	0.2 mg
d-Biotin	0.05 mg
$(NH_4)_2SO_4$	5 g
$Na_2HPO_4 \cdot 12H_2O$	7.1g
KH_2PO_4	1.36g
Agar	15g

(2) Confirmation of capability of L-glutamic acid-production of 4FG-resistant mutant strains:

The capability of glutamic acid-production of about 50 mutant strains obtained in above (1) and parent AJ13029 strain were confirmed as described below.

AJ13029 and mutant strains were each cultured on CM2B agar medium at 31.5℃ for 20 to 30 hours. A liquid medium having a composition shown as "medium A" in Table 4 was inoculated with the cells thus obtained, and the shaking culture was started at 31.5℃. About 22 hours after, the fresh medium was added so that the final concentration would be that of medium B shown in Table 4. The temperature was shifted to 37℃ and then the culture was continued further for about 24 hours. After the completion of the culture, the culture was examined with a Biotic Analyzer (a product of Asahi Chemical Industry Co., Ltd.) to determine whether L-glutamic acid was produced or not. It was thus found that when the 50 strains were cultured, two strains having a yield of glutamic acid higher than that obtained from the parent strains and a high GDH activity were separated (strain A and strain B). GDH activity of each of them was determined to find that the specific GDH activity of both of them was increased (Table 5). The GDH activity was determined by the method of E. R. Bormann et al. [Molecular Microbiol., 6, 317-326 (1996)]. By sequencing the GDH genes, it was identified that the mutation points were found only in the promoter region of GDH (Table 6).

Table 4

Ingredient	Medium A	Medium B
Glucose	3 g/dl	5 g/dl
KH_2PO_4	0.14 g/dl	0.14 g/dl
$MgS0_4 \cdot 7H_2O$	0.04 g/dl	0.04 g/dl
$FeSO_4 \cdot 7H_2O$	0.001 g/dl	0.001 g/dl
$MnSO_4 \cdot 4H_2O$	0.001 g/dl	0.001 g/dl
$(NH_4)_2SO_4$	1.5 g/dl	2.5 g/dl
Soybean protein hydrolyzate solution	1.5 ml/dl	0.38 ml/dl
Thiamine hydrochloride	0.2 mg/l	0. 2 mg/l
Biotin	0.3 mg/l	0.3 mg/l
Antifoaming agent	0.05ml/l	0.05ml/l
$CaCO_3$	5 gd/l	5 gd/l
pH	7.0(adjusted with KOH)	

Table 5. Glutamic acid formation and GDH activity of mutant strains

Strain	Glu(g/dl)	GDH specific activity	Relative value
AJ13029	2.6	7.7	1.0
FGR1	2.9	23.1	3.0
FGR2	3.0	25.9	3.4

Table 6. DNA sequences in GDH promoter region of mutant strains

Strain	GDH promoter sequence		
	-35		-10
AJ13029	TGGTCA	TTCTGTGCGACACTGC	CATAAT
FGR1	TGGTCA	TTCTGTGCGACACTGC	TATAAT

FGR2	TTGTCA	T-CTGTGCGACACTGC	TATAAT

Example 3 Introduction of mutation into CS gene promoter region of coryneform glutamate-producing bacterium:

In this Example, a strain having an enhanced promoter for the genes which codes glutamate dehydrogenase (GDH) and citrate-synthesizing enzyme (CS) was produced.

(1) Cloning of gltA gene:

The sequence of gltA gene of a coryneform bacterium, which codes citrate-synthesizing enzyme, has already been elucidated [Microbial. 140, 1817-1828 (1994)]. On the basis of this sequence, primers shown in Seq ID No. 7 and Seq ID No. 8 were synthesized. On the other hand, chromosomal DNA from Brevibacterium lactofermentum ATCC13869 was prepared using Bacterial Genome DNA Purification Kit (Advanced Genetic Technologies Corp.). Sterilized water was added to a mixture of 0.5 μg of the chromosomal DNA, 10 pmol of each of the oligonucleotides, 8μl of dNTP mixture (2.5 mM each), 5μl of 10xLa Taq Buffer (Takara Shuzo Co., Ltd.) and 2 U of La Taq (Takara Shuzo Co., Ltd.) to obtain 50μl of PCR-reaction cocktail. The reaction cocktail was subjected to PCR. The PCR conditions were 30 cycles of denaturation at 94℃ for 30 seconds, annealing at 55℃ for 15 seconds and extention at 72℃ for 3 seconds using Thermal Cycler TP240 (Takara Shuzo Co., Ltd.) to amplify about 3 Kbp of DNA fragments containing gltA gene and promoter thereof. The amplified fragements thus obtained were purified with SUPRECO2 (Takara Shuzo Co., Ltd.) and then blunt-ended. The blunting was conducted with Blunting Kit of Takara Shuzo Co., Ltd. The blunt-ended fragment was mixed with pHSG399 (Takara Shuzo Co., Ltd.) completely digested with SmaI to conduct the ligation. The ligation reaction was conducted with DNA Ligation Kit ver 2 (Takara Shuzo Co., Ltd.). After the completion of the ligation, the transformation was conducted with competent cells of E. coli JM109 (Takara Shuzo Co., Ltd.). The cells were spread on an L medium plates (comprising 10 g/1 of bactotryptone, 5 g/l of

bactoyeast extract, 5 g/l of NaCl and 15 g/l of agar; pH 7.2) containing 10 μg/ml of IPTG (isopropyl-β-D-thiogalactopyranoside), 40μ g/ml of X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) and 40 μg/ml of chloramphenicol. After culturing them overnight, white colonies were taken to obtain the transformed strains after single colony isolation.

From the transformed strains, plasmids were prepared by the alkali method (*Seibutsu Kogaku Jikken-sho* edited by Nippon Seibutsu Kogaku-kai and published by Baifukan, p. 105, 1992). Restriction enzyme maps were prepared, and the plasmid which has the same restriction map as the map shown in Fig. 2 was named "pHSG399CS".

(2) Introduction of mutations into gltA promoter:

Mutan-Super Express Km (Takara Shuzo Co., Ltd.) was used for the introduction of mutation into gltA promoter region. The method is speciffically described below. PHSG399CS was completely digestied with EcoRI and SalI to obtain EcoRI-SalI fragment containing gltA genes, which were ligated to the fragment obtained by complete digestion of pKF19kM (Takara Shuzo Co., Ltd.) with EcoRI and SalI. After the completion of the ligation, the transformation was conducted with competent cells of E. coli JM109 (Takara Shuzo Co., Ltd.). The cells was spread on L medium plates containing 10 μg/ml of IPTG, 40μg/ml of X-Gal and 25 μg/ml of kanamycin. After overnight incubation, white colonies were taken and transformants were obtained by single colony isolation. From the transformants, plasmids were prepared and the plasmid containing gltA gene was named pKF19CS.

PCR was conducted by using pKF19CS as the template and 5'-phosphorylated synthetic DNA shown in sequence of Seq ID No. 9, Seq ID No.10 and Seq ID No.11 together with the selection primer from Mutan super Express Km. The transformation was conducted with competent cells of E. coli MV1184 (Takara Shuzo Co., Ltd.) by using the PCR product. The cells were spread on L-medium plates containing 25 μg/ml of kanamycin. After overnight incubation, colonies were taken and the transformants were obtained after single colony isolation. From the

transformants, plasmid DNA was prepared. The sequence of gltA promoter region was determined by Sanger method [J. Mol. Biol., 143,161 (1980)] using synthetic DNA having the sequence of Seq ID No. 12. Specifically, the sequence was determined with a Dye Terminator Sequencing Kit (Applied Biosystems) and analyzed by Genetic Analyzer ABI310 (Applied Biosystems). The plasmids in which gltA promoter region was replaced with the sequence shown in Table 7 were named pKF19CS1, pKF19CS2 and pKF19CS4, respectively.

Table 7

	-35 region	-10 region	
pKF19CS	ATGGCT	TATAGC	
pKF19CS1	ATGGCT	TATAAC	
pKF19CS2	ATGGCT	TATAAT	pKF19CS4
	TTGACA	TATAAT	

(3) Construction of mutant gltA plasmid:

pKF19CS, pKF19CS1, pKF19CS2 and pKF19CS4 constructed in step (2) were completely digested with SalI and EcoRI (Takara Shuzo Co., Ltd.). On the other hand, plasmid pSFK6 (Japanese Patent Application No.11-69896) having a replication origin derived from plasmid pAM330 which can autonomously replicate in a coryneform bacterium [Japanese Patent Publication for Opposition Purpose (hereinafter referred to as "J. P. KOKAI") No. 58-67699] was completely digested with EcoRI and SalI. The obtained fragment was ligated with about the 2.5 kb fragment containing gltA. After the completion of the ligation, transformation was conducted with competent cells of E. coli JMI09. The cells was spread on the L-medium plates containing 10 μg/ml of IPTG, 40 g/ml of X-Gal and 25 μg/ml of kanamycin. After overnight incubation, colonies were taken and the transformants were obtained after single colony isolation. From the transformants, plasmids were prepared. The

plasmids containing gltA gene were named pSFKC, pSFKC1, pSFKC2 and pSFKC4, respectively.

(4) Determination of CS expression from mutant gltA plasmid in coryneform bacterium:

The plasmid constructed in above step (3) was introduced into Brevibacterium lactofermentum ATCC13869. Specifically, this treatment was conducted by electrical pulse method (J. P. KOKAI No. 2-07791). The transformants were selected at 31℃ with CM2B medium plate (comprising 10 g/1 of bactotryptone, 10 g/1 of bactoyeast extract, 5 g/1 of NaCl, 10μg/l of biotin and 15 g/1 of agar; pH 7.0) containing 25 μg/ml of kanamycin. After incubating for two days, colonies were taken and the transformants containing pSFKC, pSFKC1, pSFKC2 and pSFKC4 were named BLCS, BLCS1, BLCS2 and BLCS4, respectively, after single colony isolation. A medium having a composition shown in Table 8 was inoculated with the transformant. The culture was continued at 31℃ and terminated before the glucose had been completely consumed. The culture liquid was centrifuged to separate the cells. The cells were washed with 50 mM tris buffer solution (pH 7.5) containing 200m of sodium glutamate and then suspended in the same buffer solution. After the sonication with UD-201 (TOMY) followed by the centrifugation (10,000g), the cells remaining unbroken were removed to obtain a crude enzyme solution. The activity of citrate synthase can be determined according to Methods Enzymol. 13, 3-11 (1969). Specifically, the crude enzyme solution was added to a reaction mixture containing 100 mM of TisHCl. (pH 8), 0.1 mM, of DTNB, 200 mM of sodium glutamate and 0.3 mM of acetyl CoA, and the background was determined as the increase in the absorbance at 412 nm at 30℃ determined by Hitachi spectrophotometer U-3210. Further, oxaloacetic acid was added in such an amount that the final concentration thereof would be 0.5 mM. The increase in the absorbance at 412 nm was determined, from which the background value was deducted to determine the activity of the citrate synthase. The protein concentration in the crude enzyme solution was determined by Protein Assay (BIO-RAD.). Bovine serum albumin was used as the

standard protein. The results are shown in Table 9. It was confirmed that the citrate synthase activity of mutant gltA promoters was increased compared to wild-type gltA promoter.

Table 8

Ingredient	Concentration
Glucose	50 g/l
KH_2PO_4	1 g/l
$MnSO_4 \cdot 7H_2O$	0.4 mg/l
$FeSO_4 \cdot 7H_2O$	10 mg/l
Soybean protein hydrolysates	20 ml/l
Biotin	0.5 mg/l
Thiamine hydrochloride 2 mg/l	2 mg/l

Table 9

Strain	dABS/min/mg	Relative activity	Relative activity
Wild type4	6.8	1.0	
BLCS00	38.8	5.7	1.0
BLCS01	57.1	8.4	1.21
BLCS02	92.5	13.6	1.9
BLCS04	239.5	35.2	4.8

(5) Introduction of mutant gltA gene into temperature-sensitive plasmid:

For integrating mutant gltA promoter sequences into a chromosome, a method is known wherein a plasmid of which replication in a coryneform bacterium is temperature sensitive is used(J. P. KOKAI No. 5- 7491). PSFKT2 (Japanese Patent Application No.11-81693) was used as the plasmid vector, the replication of which in a coryneform bacterium is temperature sensitive. pKFCS1, pKFCS2 and pKFCS3

completely digested with SalI and BstPI and blunt-ended were used as the mutant gltA promoter sequences. They were ligated to pSFKT2 completely digested with SmaI. After the completion of the ligation, the transformation was conducted with competent cells of E. coli JM109 (Takara Shuzo Co., Ltd.). The cells were spread on the L-medium plates containing 10 μ g/ml of IPTG, 40 μ g/ml of X-Gal and 25 μ g/ml of kanamycin. After overnight incubation, white colonies were taken and the transformants were obtained after single colony isolation. From the transformants, plasmids were prepared. Temperature-sensitive shuttle vectors containing gltA gene were named pSFKTC1, pSFKTC2 and pSFKTC4, respectively.

(6) Introduction of mutant gltA promoter into chromosome:

pSFKTC1, pSFKTC2 and pSFKTC4 were each introduced into Brevibacterium lactofermentum FGR2 strain by electrical pulse method. The transformants were selected on CM2B medium plates containing 25 μ g/ml of kanamycin at 25℃. After introduction of each plasmid, each obtained strain was cultured in CM2B liquid medium, spread on CM2B plates containing 25 μ g/ml of kanamycin, after the dilution to a concentration of 10^3 to 10^5 cfu per plate and cultured at 34℃. The strain having the temperature-sensitive plasmid became sensitive to kanamycin because the replication of the plasmid was inhibited at this temperature and, therefore, it could not form colonies. On the other hand, the strain having plasmid DNA integrated into the chromosome could be selected because it formed the colonies. Colonies thus obtained were taken and separated into respective colonies. Chromosomal DNA was extracted from the strain. PCR was conducted by using the chromosomal DNA as the template and primers of sequence shown in Seq ID No. 8 and Seq ID No. 13. About 3 kb of amplified fragments were confirmed. It was thus proved that in this strain, mutant gltA gene derived from the temperature-sensitive plasmid was integrated near gltA gene in the host chromosome by homologous recombination. Strains derived from pSFKTC1, 2 and 4 were named BLCS11, BLCS12 and BLCS14, respectively.

(7) Preparation of substituted gltA promoters:

First, kanamycin-sensitive strains were obtained from the strains BLCS11, BLCS 12 and BLCS14 having mutant gltA gene integrated therein by homologous recombination. The strains having plasmid integrated therein were diluted and spread on CMM2B plates and then cultured at 34℃. After the formation of colonies, the replicas of the plates were made using CM2B plates containing 25 μg/ml of kanamycin and were cultured at 34℃. Thus, kanamycin-sensitized strains were obtained.

The chromosome was extracted from the kanamycin sensitive strain, and PCR was conducted with primers having the sequence shown in Seq ID No. 7 and Seq ID No.8 to prepare gltA gene fragments. The amplified fragments thus obtained were purified with SUPRECO2 (Takara shuzo Co., Ltd.) and then subjected to the sequencing reaction using a primer of Seq ID No. 13 to determine the sequence in the promoter region thereof. As a result, the strain having the same promoter sequence as that of pKF19CS1 in Table 7 was named GB01, the strain having the same promoter sequence as that of pKF19CS2 was named GB02 and the strain having the same promoter sequence as that of pKF19CS4 was named GB03. It was indicated that In these strains, the gltA gene of wild type originally located on the chromosome was excised together with the vector plasmid while the mutant gltA gene introduced by the plasmid was remained on the chromosome when the plasmid and duplicated gltA gene were excised from the chromosome.

(8) Determination of activity of citrate synthase of mutant gltA promoter strains:

The activities of the citrate synthase were determined by treating FGR2, GB01, GB02, GB03 and FGR2/pSFKC strains obtained in step (7) in the same manner as that of step (4). The results are shown in Table 10. It was confirmed that the citrate synthase activity of the substitited gltA promoter strain was higher than that of the parent strains thereof.

Table 10

Strain	dABS/min/mg	Relative activity
FGR2	7.9	1.0
GB01	9.5	1.2
GB02	15.0	1.9
GB03	31.6	4.0
FGR2/pSFKC	61.6	7.8

(9) Results of culture of substituted gltA promoter strains:

Each of the strains obtained in above-described step (7) was inoculated into a seed culture medium having a composition shown in Table 11, and the culture was shaked at 31.5℃ for 24 hours. 300 ml of a main culture medium having a composition shown in Table 11 was placed into 500 ml glass jar fermenters and then sterilized by heating and was inoculated by 40 ml of the seeds cultured as described above. The culture was started at a culture temperature of 31.5℃ while the stirring rate and the aeration rate were controlled at 800 to 1300 rpm and 1/2 to 1/1 vvm, respectively. The pH of the culture liquid was kept at 7.5 with gaseous ammonia. The temperature was shifted to 37℃ 8 hours after the initiation of the culture. The culture was terminated when glucose had been completely consumed in 20 to 40 hours, and the quantity of L-glutamic acid formed and accumulated in the culture liquid was determined.

As a result, the larger improvement in the yield of L-glutamic acid was confirmed when each of the strains GB02 and GB03 rather than GB01 and FGR2/pSFKC was used as shown in Table 12. From these facts, it was found that good results were obtained by introducing the mutation into the gttA promoter to increase the CS activity to 2 to 4 times for the improvement in the yield of glutamic acid produced by those strains.

Table 11

Ingredient	Concentration Seed culture	Main culture
Glucose	50 g/l	150 g/l
KH_2PO_4	1 g/l	2 g/l
$MgSO_4 7H_2O$	0.4 g/l	1.5 g/l
$FeSO_4 7H_2O$	10 mg/l	15 mg/l
$MnSO_4 4H_2O$	10 mg/l	15 mg/l
Soybean protein hydrolyzate	20 ml/l	50 ml/l
Biotin	0.5 mg/l	2 mg/l
Thiamine hydrochloride	2 mg/l	3 mg/l

Table12

Strain	L-glutamic acid (g/l)
FGR2	8.9
GB01	9.1
GB02	9.4
GB03	9.4
FGR2/pSFKC	9.1

Example 4 Introduction of mutation into ICDH gene promoter region of coryneform glutamate-producing bacterium:

In this Example, strains having enhanced promoters for genes which codes glutamate dehydrogenase, citrate synthase and isocitrate dehydrogenase were produced.

(1) Cloning of icd gene:

The DNA sequence of icd gene of coryneform bacterium, which codes citrate synthase, has already been elucidated [J. Bacteriol. 177, 774-782 (1995)]. On the

bases of this sequence, primers shown in Seq ID No. 14 and Seq ID No.15 were synthesized. PCR was conducted by using chromosomal DNA of Brevibacterium lactofermentum ATCC13869 as the template to amplify about 3 Kbp of DNA fragment containing icd gene and promoter thereof. The amplified fragment thus obtained was completely digested with EcoRI, and mixed with that obtained by complete digestion of pHSG399 (Takara Shuzo Co., Ltd.) with EcoRI to conduct the ligation. After the completion of the ligation, the transformation was conducted using competent cells of E. coli JM109. The cells were spread on the L-medium plates containing 10 μg/ml of IPTG, 40μg/ml of X-Gal and 40μg/ml of chloramphenicol After overnight incubation, white colonies were taken and the transformants was obtained after single colony isolation.

The plasmid carrying icd gene was named pHSG399icd.

(2) Introduction of mutations into icd promoter:

The accurate location of the promoter of icd gene has not yet been determined. The possibility of increasing mRNA transcription level of icd gene was investigated by artificially modifying the upstream sequence of the gene which codes ICDH into a promoter-like sequence. Specifically, mutations were introduced into the −10 like region existing in the DNA sequence about 190 bp upstream (the first promoter) and about 70 bp (the second promoter) upstream from the first ATG of ICDH protein.

Mutan-Super Express Km (Takara Shuzo Co., Ltd.) was used for the introduction of mutation into an upstream region of icd gene. The method is specifically described below. pHSG399icd was completely digested with PstI to obtain PstI fragment containing the promoter of icd gene. The fragments were ligated with the fragment obtained by complete digestion of pKF18kM (Takara Shuzo Co., Ltd.) with PstI. After the completion of the ligation, the transformation was conducted with competent cells of E. coli JM109 (Takara Shuzo Co., Ltd.). The cells was spread on the L-medium containing 10 μg/ml of IPTG, 40μg/ml of X-Gal and 25

μg/ml of kanamycin. After overnight incubation, white colonies were taken and transformants were obtained after single colony isolation. From the transformants, plasmids were prepared, and the plasmid containing the promoter of icd gene was named pKF18icd.

PCR was conducted by using pKF18icd as the template and 5'-phosphorylated synthetic DNA shown in Seq ID No. 16, Seq ID No.17, Seq ID No.18, Seq ID No.19, Seq ID No.20 and Seq ID No.21 and the selection primer. These PCR products were used for transforming competent cells of E. coli JM109. The cells were spread on the L-medium plates containing 25μg/ml of kanamycin. After overnight incubation, formed colonies were taken and the transformants were obtained after single colony isolation. From the transformants, plasmid DNA was prepared, and the sequence of icd promoter region was determined using synthetic DNA shown in Seq ID No. 22 by Sanger's method [J. Mol. Biol., 143, 161 (1980)]. Specifically, the DNA sequence was determined with Dye Terminator Sequencing Kit (Applied Biosystems), and analyzed with Genetic Analyzer ABI310 (Applied Biosystems). Those obtained by replacing icd promoter region with a sequence shown in Table 7 were named pKF18ICD1, pKF18ICD2, pKF18ICD3, pKF18ICD4, pKF18ICD5 and pKF18ICD6m respectively. Among them, pKF18ICD2 was completely digested with PstI to obtain PstI fragment containing the promoter of icd gene. The fragment was ligated with the fragment obtained by complete PstI digestion of pKF18kM (Takara Shuzo Co., Ltd.). After the completion of the ligation, the transformation was conducted with competent cells of E. coli JM109 (Takara Shuzo Co., Ltd.). The cells were spread on the L-medium plates containing 10 μg/ml of IPTG, 40μg/ml of X-Gal and 25μg/ml of kanamycin. After overnight incubaton, white colonies were taken and the transformed strains were obatined after single colony isolation. From the transformed strains, plasmids were prepared, and the plasmid containing the promoter of icd gene was named pKF18ICDM2. PCR was conducted using pKF18ICDM2 as the template and 5'-phosphorilated synthetic DNA shown in Seq ID No. 20 and Seq ID No.21 and the selection primer. The

transformation of competent cells of E. coli JM109 was conducted with the PCR product. The cells were spread on the L-medium plates containing 25μg/ml of kanamycin. After overnight incubation, colonies thus formed were taken and transformants were obatined after single colony isolation. From the transformants, plasmids DNA were prepared, and the sequence of icd promoter region was determined using synthetic DNA shown in Seq ID No. 22. Those obtained by replacing icd promoter region with the sequence shown in Table 13 were named pKF18ICD25 and pKF18ICD26, repectively.

Table 13

Plasmid	1st Promoter		2nd Promoter	
	-35	-10	-35	-10
pKF18ICD	GCGACT	GAAAGT	TTTCCA	CACCAT
pKF18ICD01	GCGACT	TATAAT	TTTCCA	CACCAT
pKF18ICD02	TTGACA	TATAAT	TTTCCA	CACCAT
pKF18ICD03	TTGACT	TAAAGT	TTTCCA	CACCAT
pKF18ICD04	GCGACT	GAAAGT	TTTCCA	TATAAT
pKF18ICD05	GCGACT	GAAAGT	TTGCCA	TATAAT
pKF18ICD06	GCGACT	GAAAGT	TTGACA	TATAAT
pKF18ICD25	TTGACA	TATAAT	TTGCCA	TATAAT
pKF18ICD26	TTGACA	TATAAT	TTGACA	TATAAT

(3) Plasmid construction for determination of promoter activity:

For easily determining the promoter activity, a possible method is the indirect determination of the promoter activity using a reporter gene. Desirable properties required of the reporter gene are that the activity can be easily determined, that even when an amino acid is added to an N-terminal, the activity is not seriously lowered, that the background reaction does not occur and that it has a restriction enzyme

cleavage site suitable for the gene manipulation. Because β galactosidase (LacZ) of E. coli is widely used as a reporter gene and bacteria of the genus Corynebacterium do not have lactose assimilability [J. Gen. Appl. Microbiol., 18, 399-416 (1972)], LacZ was determined to be the optimum reporter gene. Then, plasmid pNEOL carrying LacZ as the reporter gene was constructed (see Fig.3). The process for the construction is described in detail below. PCR was conducted by using a chromosomal DNA obtained from E coli ME8459(ME8459 was deposited with National Institute of Genetics (Japan)) as the template with synthetic DNA shown in Seq ID No. 23 and Seq ID No.24 as the primer. The PCR product was completely digested with SmaI and BamHI and then ligated with fragments obtained by digesting pKF3 (Takara Shuzo Co., Ltd.) with HindIII and blunt-ended. After the completion of the ligation, the transformation was conducted with competent cells of E. coli JM109 (Takara Shuzo Co., Ltd.). The cells were spread on the L-medium plates containing 25 μ g/ml of kanamycin. After overnight incubation, colonies thus formed were taken and separated into respective colonies to obtain the transformed strain. The plasmid obtained from the transformed strain was named pKF3nptII. Then, this plasmid was digested with SalI. On the other hand, pSAK4 described in Example 1(1) was completely digested with SmaI and SalI and blunt-ended. These fragments were ligated together to construct a shuttle vector pNEO which can replicate in a coryneform bacterium. This plasmid was capable of imparting a resistance to chloramphenicol and resistance to kanamycin to the hosts. Further, pNEO was completely digested with SmaI and Sse8387I. The resultant fragments were ligated to those obtained by complete digestion of pMC1871 (Farmacia Biotech.) with PstI and SmaI. Thus, shuttle vector pNEOL which can be replicated in a coryneform bacterium and having LacZ lacking 8 amino acid on N-terminal as the reporter gene was constructed (see Fig.3).

(4) Determination of activity of mutant icd promoter:

Plasmids having mutant icd promoter constructed in above-described step (2),

i. e. pKF18ICD1, pKF18ICD2, pKF18ICD3, pKF18ICD4, pKF18ICD5, pKF18ICD6, pKF18ICD25, pKF18ICD26 and pKF18ICD, were completely digestede with SacII and PstI and then blunt-ended. They were ligated with fragment obtained by digesting pNEOL with SmaI. After the completion of the ligation, the transformation was conducted with competent cells of E. coli JM109. The cells were spread on the L-medium plates containing IPTG, X-Gal and 40 μ g/ml of chloramphenicol. After overnight incubation, blue colonies were taken and the transformed strains were obtained after single colony isolation.

From the transformed strains, plasmids were prepared. Plasmids having a structure capable of producing a fused protein of ICDH and LacZ were named pNEOICD1, pNEOICD2, pNEOICD3, pNEOICD4, pNEOICD5, pNEOICD6, pNEOICD25, pNEOICD26 and pNEOLICD, respectively. Each of These plasmids or pNEOL was introduced into Brevibacterium lactofermentum ATCC13869 by electrical pulse method. The transformants were selected by using CM2B medium plates (comprising 10 g/l of bactotryptone,10 g/l of bactoyeast extract, 5 g/l of NaCl, 10 μ g/l of biotin and 15 g/l of agar and having pH 7.0) containing 25 μ g/ml of kanamycin and 40 μ g/ml of X-Gal at 31℃ for two days. After the completion of the introduction, colonies thus formed were taken and isolated as single colonies. The transformants containing pNEOICD1, pNEOICD2, pNEOICD3, pNEOICD4, pNEOICD5, pNEOICD6, pNEOICD25, pNEOICD26 and pNEOLICD were named BLAC1, BLAC2, BLAC3, BLAC4, BLAC5, BLAC6, BLAC25, BLAC26, BLAC and BNEOL, respectively. All the transformants other than BNEO formed blue colonies. Crude enzyme solutions were prepared from the transformants in the same manner as that of step (4) in Example 3 except that "Z-Buffer" (comprising 10 mM of KCl, 1 mM of $MgSO_4$, 270 μ g/100 mM of 2-ME and NaPi and having pH 7.5) was used as a washing and suspension buffer. The activity of LacZ was determined as follows: Z-Buffer was mixed with the crude enzyme solution, ONPG in Z-Buffer having the final concentration of 0.8 mg/ml was added to the resultant mixture, and the increase in the absorbance at 420 nm at 30℃ was determined with Hitachi spectrophotometer U-3210 as the activity of LacZ. The

protein concentration in the crude enzyme solution was determined by Protein Assay (BIO-RAD). Bovine serum albumin was used as the standard protein. The results are shown in Table 14. It was confirmed that the LacZ activity of the strain having a mutation in icd promoter and expressing ICDH-LacZ fused protein was higher than that expressing the wild type ICDH-LacZ fused protein.

Table 14

Strain	dABS/min/mg	Relative activity
BNEOL	Not detected	0.0
BNEOLI	42	1.0
BNEOLI-1	84	2.0
BNEOLI-2	168	4.0
BNEOLI-3	80	1.9
BNEOLI-4	126	3.0
BNEOLI-5	139	3.3
BNEOLI-6	84	2.0
BNEOLI-25	168	4.0
BNEOLI-26	170	4.0

(5) Introduction of mutant icd gene into temperature-sensitive plasmid:

Plasmid vector pSFKT2 (Japanese Patent Application No. 11-81693) the replication of which in a coryneform bacterium was temperature-sensitive was used. pKF18ICD1, pKF18ICD2, pKF18ICD3, pKF18ICD4, pKF18ICD5, pKF18ICD6, pKFICD25 and pKFICD26 were completely digested with PstI and the obtained fragments were used as the mutant icd promoter sequences. The fragments thus obtained were ligated with pSFKT2 completely digested with PstI. After the completion of the ligation, the transformation was conducted with competent cells of E. coli JM109 (Takara Shuzo Co., Ltd.). The cells were spread on the L-medium plates

containing 10 μ g/ml of IPTG, 40 μ g/ml of X-Gal and 25 μ g/ml of kanamycin. After overnight incubation, white colonies were taken and transformed strains were obtained after single colony isloation. From the transformed strains, plasmids were prepared. Temperature-sensitive shuttle vectors containing icd promoter were named pSFKTI1, pSFKTI2, pSFKTI3, pSFKTI4, pSFKTI5, pSFKTI6, pSFKTI25 and pSFKTI26, respectively.

(6) Integreation of mutant icd promoter into chromosome:

The plasmids constructed in above-described step (5) were each introduced into Brevibacterium lactofermentum GB02 strain by electrical pulse method. The transformants were selected with CM2B medium plates(comprising 10 g/l of bactotryptone,10 g/l of bactoyeast extract, 5 g/l of NaCl, 10 μ g/l of biotin and 15 g/l of agar and having pH 7.0) containing 25 μ g/ml of kanamycin at 25℃. After the completion of the introduction, the obtained strains were cultured in CM2B liquid medium, spread on CM2B plates containing 25 μ g/ml of kanamycin after the dilution to a concentration of 10^3 to 10^5 cfu per plate and cultured at 34℃. The strain having the temperature-sensitive plasmid became sensitive to kanamycin because the replication of the plasmid was inhibited at this temperature and, therefore, it could not form colonies. On the other hand, the strain having plasmid DNA integrated into the chromosome could be selected because it could form colonies. Colonies thus obtained were taken and separated into isolated colonies. Chromosomal DNA was extracted from the strain and PCR was conducted by using the chromosomal DNA as the template with primers shown in Seq ID No. 13 and Seq ID No. 15. About 3 kb of amplified fragments were confirmed. It was thus proved that in this strain, mutant icd gene derived from the temperature-sensitive plasmid was integrated near icd gene in the host chromosome by homologous recombination.

(7) Preparation of strains having substituted icd promoter:

First, kanamycin-sensitive strain was obtained from the strains having mutant icd gene integrated therein by the homologous recombination as described in step (6).

The strains having the plasmid integrated therein were diluted and spread on CM2B plates and then cultured at 34℃. After the formation of colonies, replicas were made on CM2B plates containing 25 μ g/ml of kanamycin, and they were incubated at 34℃. Thus, kanamycin-sensitive strains were obtained.

The chromosome was extracted from the kanamycin resistant strain, and PCR was conducted using primers shown in Seq ID No.14 and Seq ID No.15 to prepare icd gene fragments. The amplified fragments thus obtained were purified with SUPRECO2 (Takara shuzo Co., Ltd.) and then subjected to the sequencing reaction using a primer shown in Seq ID No. 22 to determine the sequence of the promoter region thereof. As a result, strains having icd promoter sequences derived from pSFKTI1, pSFKTI2, pSFKTI3, pSFKTI4, pSFKTI5, pSFKTI6, pSFKTI25 and pSFKTI26 were named GC01, GC02, GC03, GC04, GC05, GC06, GC25 and GC26, respectively. In these strains, when the plasmid and duplicate icd gene were excised from the chromosome, the icd gene of wild type originally located on the chromosome was excised together with the vector plasmid, while the mutant icd gene introduced by the plasmid remained on the chromosome.

(8) Determination of isocitrate-dehydrogenase activity of the mutant strains having mutant icd promoter:

ICDH crude enzyme solution was prepared by using each of the 8 strains obtained in above-described step (7) and GB02 strain in the same manner as that of step (7) in Example 3. The ICDH activities were determined as follows: The crude enzyme solution was added to a reaction solution containing 35 mM of TisHCl (pH 7.5), 1.5 mM of $MnSO_4$, 0.1 mM of NADP and 1.3 mM of isocitric acid, and the increase in the absorbance at 340 nm at 30℃ was determined with Hitachi spectrophotometer U-3210 as the activity of ICDH. The protein concentration in the crude enzyme solution was determined by Protein Assay (BIO-RAD). Bovine serum albumin was used as the standard protein. The results are shown in Table 15. It was confirmed that the isocitrate dehydrogenase activity of substituted icd promoter strains was higher than

that of the parent strain.

Table 15

Strain	dABS/min/mg	Relative activity
GB02	3.9	1.0
GC01	8.2	2.1
GC02	19.1	4.9
GC03	7.0	1.8
GC04	12.5	3.2
GC05	19.1	4.9
GC06	10.5	2.7
GC25	30.4	7.8
GC26	24.2	6.2

(9) Results of culturing the strains containing substituted icd promoter:

Each of the 8 strains obtained in above-described step (7) was cultured in the same manner as that in step (9) in Example 3. As a result, the improvement in the yield of L-glutamic acid was confirmed when any one of the strains GC02, GC04, GC05, GC25 and GC26 was used as shown in Table 16. It was found that good results were obtained by introducing the mutation into icd promoter to increase the ICDH activity to at least 3 times.

Table16

Strain	L-glutamic acid (g/dl)
GB02	9.2
GC01	9.0
GC02	9.5
GC03	9.1

GC04	9.4
GC05	9.6
GC06	9.2
GC25	9.9
GC26	9.8

Example 5 Introduction of mutation into PDH gene promoter region of coryneform glutamate-producing bacterium:

(1) Cloning of pdhA gene from coryneform bacteria

Primers shown in Seq ID No.25 and Seq ID No.26 were synthesized by selecting regions having a high homology among EI subunits of pyruvate dehydrogenase (PDH) of Escherichia coli, Pseudomonas aeruginosa and Mycobacterium tuberculosis. PCR was conducted by using chromosome of Brevibacterium lactofermentum ATCC13869, prepared with a bacterial genomic DNA purification kit (Advanced Genetic Technologies Corp.), as the template under standard reaction conditions described on page 8 of PCR Technology (edited by H. Erlich and published by Stockton Press, 1989). The reaction solution was subjected to the electrophoresis in an agarose gel to find that about 1.3 kilobases of DNA fragment was amplified. The sequence of both end of the obtained DNA was determined with synthetic DNA shown in Seq ID No. 25 and Seq ID No.26. The sequence was determined by Sanger's method [J. Mol. Biol., 143, 161 (1980)] with DNA Sequencing Kit (Applied Biosystems Co.). The determined sequence was deduced to amino acids, and compared with E1 subunits of pyruvate dehydrogenase derived from each of Escherichia coli, Pseudomonas aeruginosa and Mycobacterium tuberculosis to find a high homology among them. Consequently, it was dtermined that the DNA fragment amplified by PCR was a part of pdhA gene which codes EI subunit of pyruvate dehydrogenase of Brevibacterium lactofermentum ATCC13869. The cloning of the upstream and downstream region of the gene was conducted. The cloning method was as follows: A chromosome of Brevibacterium lactofermentum ATCC13869 was

digested with restriction enzymes EcoRI, BamHI, Hind III, Pst I, Sal I and Xba I (Takara Shuzo Co., ltd.) to obtain DNA fragments. LA PCR in vitro cloning Kit (Takara Shuzo Co., Ltd.) was used for the cloning, using the sequences shown in Seq ID No. 27 and Seq ID No.28 in the Sequence Listing as primers for cloning the upstream region, and sequences shown in Seq ID No. 29 and Seq ID No.30 as primers for cloning the downstream region. After PCR using the kit, DNA fragments of about 0.5, 2.5, 3.0, 1.5 and 1.8 kilobases were amplified for the upstream region from the fragments obtained by digestion with EcoRI, Hind III, Pst I, Sal I and Xba I, respectrively; and DNA fragments of about 1.5, 3.5 and 1.0 kilobase were amplified for the downstream region from the fragements obtained by digestion with BamHI, Hind III and Pst I, respectively. The sequences of these DNA fragments were determined in the same manner as that described above. It was found that the amplified DNA fragments further contained an open reading frame of about 920 amino acids and also that a region supposed to be a promoter region was present in the upstream region. Because the deduced amino acid sequence from the DNA sequence of the open reading frame is highly homologous to known EI subunit of pyruvate dehydrogenase such as that of E. coli, it was apparent that the open reading frame was the pdhA gene which codes EI subunit of pyruvate dehydrogenase of Brevibacterium lactofermentum ATCC13869. The DNA sequence of the open reading frame was shown in Seq ID No. 31 in the Sequence Listing. In Seq ID No. 31 in the Sequence Listing, deduced amino acid sequence from the DNA sequence is also shown. Since methionine residue at N-terminal of the protein is derived from ATG which is an initiation codon, it usually does not concern the essential function of protein, and it is well known that the methionine residue is removed by the effect of peptidase after the translation. Therefore, in the above-described protein, it is possible that methionine residue at the N-terminal has been removed. However, the GTG sequence is present in 6 bases upstream of ATG shown in Seq ID No. 31 in the Sequence Listing, and it is also possible that amino acids is translated from this point. Pyruvate dehydrogenase of other microorganisms such as E. coli are composed of

three subunits of E1, E2 and E3, and genes which encode them constitute an operon in many cases. However, there was no open reading frame considered to be E2 and E3 subunit of pyruvate dehydrogenase in about 3 kilobases downstream of pdhA gene. Instead, it was shown that a sequence supposed to be a terminator was present in the downstream of the open reading frame. From these facts, it was supposed that E2 and E3 subunits of pyruvate dehydrogenase of Brevibacterium lactofermentum ATCC13869 were present in another regon on the chromosome.

(2) Construction of a plasmid for amplifying pdhA:

It was already apparent that a strain obtained by introducing a gene which codes three subunits constituting PDH of E. coli into Brevibacterium lactofermentum ATCC13869 gives an improved glutamic acid yield (JP No.10-360619). However, in PDH of Brevibacterium lactofermentum ATCC13869, only pdhA gene which codes El subunit had been cloned, and no examination had not been made to know whether the amplification of the gene alone is effective in improving the yield of glutamic acid. Under these circumstances, examination was made to know whether the amplification of pdhA gene alone is effective in improving the yield of glutamic acid or not.

Primers shown in Seq ID No. 33 and Seq ID No.34 were synthesized on the basis of the previously cloned DNA sequences. PCR was conducted by using chromosome of Brevibacterium lactofermentum ATCC13869, prepared with a Bacterial Genomic DNA Purification kit (Advanced Genetic Technologies Corp.), as the template under standard reaction conditions described on page 8 of PCR Technology (edited by H. Erlich and published by Stockton Press, 1989) to amplify pdhA gene. Among the primers thus synthesized, Seq ID No. 33 corresponded to a sequence of base No. 1397 to No.1416 in pdhA gene described in Seq ID No. 32 in the Sequence Listing. Seq ID No. 34 was the complementary strand of the DNA sequence corresponding to the sequence of base No. 5355 to No.5374 in Seq ID No. 32 in the Sequence Listing, which was represented from the 5' side.

PCR product thus obtained was purified by an ordinary method and reacted

with restriction enzyme Sal I and EcoT22I. The fragment was ligated with pSFK(Patent Application No.11-69896), cleaved with restriction enzymes Sal I and Pst I, with a ligation kit (Takara Shuzo Co., Ltd.). After the transformation with competent cells (Takara Shuzo Co., Ltd.) of E. coli JM109, the cells were spread to the L-medium medium plates(comprising 10 g/l of bactotryptone, 5 g/l of bactoyeast extract, 5 g/l of NaCl and 15 g/l of agar and having pH 7.2) containing 10 μ g/ml of IPTG (isopropyl- β -D-thiogalactopyranoside), 40 μ g/ml of X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) and 25 μ g/ml of kanamycin. After overnight incubation, white colonies were taken and the transformed strains were obtained after single colony isolation.

From the transformned strains, plasmids were prepared by alkali method (*Seibutsu Kogaku Jikken-sho* edited by Nippon *Seibutsu Kogaku kai* and published by *Baifukan*, p. 105, 1992). Restriction enzyme maps of DNA fragments inserted into the vectors were prepared and compared with the restriction enzyme map of pdhA gene reported in sequence No. 32 of the Sequence Listing. A plasmid containing DNA fragments inserted therein having the same restriction enzyme map as that of pdhA gene was named pSFKBPDHA.

(3) Introduction of pASFKBPDHA into Brevibacterium lactofermentum ATCC13869 and GC25 and evaluation of the fermentation experiments:

Brevibacterium lactofermentum ATCC13869 and GC25 were transformed with plasmid pSFKBPDHA by electrical pulse method (J. P. KOKAI No.2-207791) to obtain the transformed strains. The culture for producing L-glutamic acid was conducted with transformed strain ATCC13869/pSFKBPDHA and GC25/pSFKBPDHA obtained by introducing plasmid pSFKBPDHA into Brevibacterium lactofermentum ATCC13869 and GC25 as follows: Cells of ATCC13869/pSFKBPDHA and GC25/pSFKBPDHA obtained by the culture on CM2B medium plates containing 25 μ g/ml of kanamycin were inoculated into a medium (comprising 1 liter of pure water containing 80 g of glucose, 1 g of KH_2PO_4, 0.4 g of $MgSO_4 7H_2O$, 30 g of $(NH_4)_2SO_4$, 0.01 g of $FeSO_4$

$7H_2O$, 0.01 g of $MnSO_4$ $7H_2O$, 15 ml of soybean protein hydrolyzate, 200 μg of thiamine hydrochloride, 60 μg of biotin, 25 mg of kanamycin and 50 g of $CaCO_3$; and having a pH adjusted to 8.0 with KOH). Then the culture was shaked at 31.5℃ until sugar in the medium had been consumed. The obtained products were inoculated into the medium of the same composition as that described above (for GC25/pSFK6 and GC25/pSFKBDHA) or the medium eliminated Biotin from the composition as that described above(for ATCC13869/pSFK6 and ATCC13869/pSFKBPDHA) in an amount of 5 %, and the shaking culture was conducted at 37℃ until sugar in the medium had been consumed. As a control, strains obtained by transforming Brevibacterium lactofermentum ATCC13869 and GC25 with previously obtained palsmid pSFK6 capable of autonomously replicating in coryneform bacterium by electrical pulse method (J. P. KOKAI No. 2-207791), were cultured in the same manner as that described above. After the completion of the culture, the amount of L-glutamic acid accumulated in the culture medium was determined with Biotic Analyzer AS-210 (a product of Asahi Chemical Industry Co., Ltd.). The results are shown in Table 17.

Table 17

Strain	Yield of L-glutamic acid (g/dl)
ATCC13869/Psfk	3.6
ATCC13869/pSFKBPDHA	3.8
GC25/pSFK 6	5.1
GC25/pSFKBPDHA	5.3

From these results, it was apparent that even the amplification of pdhA gene alone is sufficiently effective in improving the yield of Glu in Brevibacterium lactofermentum ATCC13869 and GC25.

(4) Construction of plasmids for determination of the activity of mutated pdhA promoter:

To produce promoter mutant of pyruvate dehydrogenase (PDH), the determination of the previously cloned promoter region of pdhA gene of Brevibacterium lactofermentum ATCC13869 was conducted and also the determination of difference in the expression caused by the modification of the promoter region were conducted by determining the activity of β-galactosidase.

The promoter region of pdhA gene was presumed from the DNA sequence which had been already elucidated by cloning. As a result, it was supposed to be possible that base No. 2252 to No.2257 and No. 2279 to No. 2284 in Seq ID No. 32 in the Sequence Listing were –35 region and –10 region, respectively. Therefore, primers shown as Seq ID No. 35 and Seq ID No.36 in the Sequence Listing were synthesized, and DNA fragments containing promoter region of pdhA gene were amplified by PCR method by using chromosomal DNA of Brevibacterium lactofermentum ATCC13869 as a template. Among the synthesized primers, Seq ID No. 35 corresponded to the sequence ranging from base No. 2194 to base No. 2221 in Seq ID No. 32; but the base No. 2198 had been replaced with C, and the base No. 2200 and No.2202 had been replaced with G, and recognition sequence for restriction enzyme SmaI had been inserted. Seq ID No. 36 corresponded to the sequence ranging from base No. 2372 to base No. 2398 in Seq ID No. 32; but base No.2393 and No.2394 had been replaced with G, and the complementary strand of the DNA sequence having a recognition sequence of restriction enzyme SmaI inserted therein was represented from the 5'-end. PCR was conducted by using chromosome of Brevibacterium lactofermentum ATCC13869, prepared with Bacterial Genomic DNA Purification kit (Advanced Genetic Technologies Corp.), as the template under standard reaction conditions described on page 8 of PCR Technology (edited by H. Erlich and published by Stockton Press, 1989) to amplify the promoter region of pdhA gene. PCR product thus obtained was purified by an ordinary method and reacted with restriction enzyme Sma I. The fragments were ligated with pNEOL lacking in

promoter region of lacZ gene which could be replicate in a coryneform bacterium and which had been digested with restriction enzymes Sma I, (Example 4 (3) with a Ligation Kit (Takara Shuzo Co., Ltd.). After the transformation with competent cells (Takara Shuzo Co., Ltd.) of E. coli JM109, the cells were spread on the L-medium plates(comprising 10 g/l of bactotryptone, 5 g/l of bactoyeast extract, 5 g/l of NaCl and 15 g/l of agar and having pH 7.2) containing 40 μ g/ml of X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) and 25 μ g/ml of kanamycin. After overnight incubation, blue colonies were taken and the transformed strains were obtained after single colony isolation. From the transformants, plasmids were prepared by alkali method (*Seibutsu Kogaku Jikken-sho* edited by *Nippon Seibutsu Kogaku-kai* and published by *Baifukan*, p. 105, 1992). After sequencing DNA fragments inserted into the vector by an ordinary method, the plasmid containing the DNA fragment inserted therein was named pNEOLBPDHAprol.

Further, primers indicated as Seq ID No. 37, Seq ID No.38 and Seq ID NO.39 in the Sequence Listing were synthesized for constructing plasmids wherein a region supposed to be the promoter site was changed to the consensus sequence of promoters of coryneform bacteria. By using each of the primers and a primer shonw in Seq ID No. 36, DNA fragments wherein the promoter region of pdhA gene was changed to the consensus sequence were amplified by PCR method by using chromosomal DNA of Brevibacterium lactofermentum ATCC13869 as a template. Among the synthesized primers, Seq ID No. 37 corresponded to the sequence ranging from base No. 2244 to base No. 2273 in Seq ID No. 32; base No. 2255 had been replaced with C, and base No. 2257 had been replaced with A; thus only –35 region had been changed to the consensus sequence of the coryneform bacteria. Seq ID No. 38 corresponded to the sequence ranging from base No. 2249 to base No. 2288 in sequence No. 32; base No. 2279 and No.2281 had been replaced with T; thus only –10 region had been changed to the consensus sequence of the coryneform bacteria. Sequence No. 39 corresponded to the sequence ranging from base No. 2249 to base No. 2288 in Seq ID No. 32; base No. 2255 had been replaced with C,

base No. 2257 had been replaced with A, and base No. 2279 and No.2281 had been replaced with T; thus both –35 region and –10 region had been changed to the consensus sequence of the coryneform bacteria. PCR was conducted by using chromosome of Brevibacterium lactofermentum ATCC13869, prepared with a Bacterial Genomic DNA Purification Kit (Advanced Genetic Technologies Corp.), as the template under standard reaction conditions described on page 8 of PCR Technology (edited by H. Erlich and published by Stockton Press, 1989) to amplify the promoter region of pdhA gene with these primers so that the promoter region was changed to the consensus sequence. PCR products thus obtained were purified by an ordinary method and reacted with restriction enzyme SmaI. The fragments were ligated with pNEOL lacking the promoter region of lacZ gene, which could replicate in a coryneform bacterium and which had been cleavaged with restriction enzymes Sma I, with a Ligation Kit (Takara Shuzo Co., Ltd.). After the transformation with competent cells (Takara Shuzo Co., Ltd.) of E. coli JM109, the cells were spread on the L-medium plates (comprising 10 g/l of bactotryptone, 5 g/l of bactoyeast extract, 5 g/l of NaCl and 15 g/l of agar and having pH 7.2) containing 40 μ g/ml of X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) and 25 μ g/ml- of kanamycin. After overnight incubation, blue colonies were taken and the transformed strains were obatined after single colony isolation. From the transformed strains, plasmids were prepared by the alkali method (*Seibutsu Kogaku Jikken-sho* edited by *Nippon Seibutsu Kogaku kai* and published by *Baifukan*, p. 105, 1992). After sequencing DNA fragments inserted into the vector by an ordinary method, the plasmid containing DNA fragments, wherein only the sequence in –35 region had been changed to the consensus sequence, inserted therein was named pNEOLBPDHApro35; the plasmid containing DNA fragments, wherein only the sequence in –10 region had been changed to the consensus sequence, was inserted therein was named pNEOLBPDHApro10; and the plasmid containing DNA fragments, wherein the sequences in both –35 region and –10 region had been changed to the consensus sequence, was inserted therein was named pNEOLBPDHApro3510.

(5) The elavuation of the mutated pdhA promoter activity:

Brevibacterium lactofermentum ATCC13869 was transformed with plasmids named pNEOLBPDHApro1, pNEOLBPDHApro10 and pNEOLBPDHApro3510 by electrical pulse method (J. P. KOKAI No. 2-207791) to obtain the transformed strains. β-galactosidase activity of the obtained transformants was determined by the method described in Example 4(4). After changing the sequence in the promoter region to the consensus sequence, β-galactosidase activities were as shown in Table 18, wherein the enzymatic activity of β-galactosidase having the promoter region of pdhA gene was given as 1.

Table 18

Strain	β-Galactosidase activity (relative value)
ATCC13869/pNEOLBPDHAproI	1
ATCC13869/pNEOLBPDHAproI0	6
ATCC13869/pNEOLBPDHApro3510	7.5

These results indicate that the supposed promoter region was the promoter of pdhA gene and that the expression of PdhA can be changed (enhanced) by changing the sequence in this region into the consensus sequence. This fact indicates that the expression can be changed, without using plasmid, by changing the promoter region of pdhA gene.

(6) Construction of plasmid for preparation of promoter varied strain:

Since it had been proved that the expression of pdhA can be changed by introducing mutations into the promoter, plasmids for preparing a pdhA promoter modified strains were constructed. Three constructs of the plasmids for the promoter modified strains were constructed. They were plasmids wherein -35 region, -10 region and both of them were changed to the consensus sequence, respectively.

Primers shown in Seq ID No. 40 and Seq ID No.41 were newly synthesized on the basis of the DNA sequence which had already been cloned. Among synthesized primers, Seq ID No. 40 was the complementary strand of the DNA sequence corresponding to a sequence ranging from base No. 2491 to base No. 2521 in Seq ID No. 32, which was represented from the 5'-end, and to which a sequence comprising three A's followed by four T's at the 5' terminal. Seq ID No. 33 was the complementary strand of the DNA sequence corresponding to the sequence ranging from base No. 5020 to base No. 5039 of pdhA gene in Seq ID No. 32, which was represented from the 5'-end. PCR was conducted by using Seq ID No. 33 and Seq ID No.40 as the primers and chromosome of Brevibacterium lactofermentum ATCC13869, prepared with Bacterial Genomic DNA Purification Kit (Advanced Genetic Technologies Corp.), as a template under standard reaction conditions described on page 8 of PCR Technology (edited by H. Erlich and published by Stockton Press. 1989). Further, PCR was conducted by using Seq ID No. 39 and Seq ID No. 41 and chromosome of Brevibacterium lactofermentum ATCC13869 as a template. The PCR products thus obtained were purified by an ordinary method. PCR was conducted by using PCR products obtained by using Seq ID No. 33 and No.40, PCR products obtained by using Seq ID No. 39 and Seq ID No.41 and Seq ID No. 33 and 41 as the primers. The PCR condition was as follows: The concentration of these four DNA would be 10 μM in the reaction cocktail and La taq (Takara Shuzo Co., Ltd.) was used without template. PCR products were purified by an ordinary method, and reacted with restriction enzyme Sal I and Xho I. The fragments thus obtained were ligated with fragments obtained by digesting temperature-sensitive plasmid pSFKT2 with SalI, which can replicate in a coryneform bacterium, by using Ligation Kit (Takara Shuzo Co., Ltd.). After the transformation with competent cells (Takara Shuzo Co., Ltd.) of E. coli JM109, the cells was spread on the L-medium plates(comprising 10 g/l of bactotryptone, 5 g/l of bactoyeast extract, 5 g/l of NaCl and 15 g/l of agar and having pH 7.2) containing 10 μg/ml of IPTG (isopropyl- β -D-thiogalactopyranoside), 40 μ g/ml of X-Gal (5-bromo-4-chloro-3-

indolyl-β-D-galactoside) and 25 μ g/ml of kanamycin. After overnight incubation, white colonies were taken and transformants were obtained after single colon isolation. From the transformants, plasmids were prepared by the alkali method (*Seibutsu Kogaku Jikken-sho* edited by *Nippon Seibutsu Kogaku-kai* and published by *Baifukan*, p. 105, 1992). After sequencing DNA fragments inserted into the vector, the base sequence was compared with that of pdhA gene reported in sequence No. 32. The plasmid containing DNA fragments, wherein only the sequences in –35 region and –10 region of the promoter were changed to the consensus sequence of the coryneform bacteria, inserted therein was named pSFKTPDHApro3510.

A plasmid wherein –35 region of the promoter of pdhA gene had been changed to the consensus sequence of coryneform bacteria, and also plasmid wherein –10 region of the promoter of pdhA gene had been changed to the consensus sequence of coryneform bacteria were constructed in the same manner as that described above except that Seq ID No. 39 in the Sequence Listing was replaced with Seq ID No. 37 and 38, respectively. These plasmids were named pSFKTPDHApro35 and pSFKTPDHApro10, respectively.

(7) Preparation of promoter modified strains:

Strains having modified pdhA gene promoter were prepared by the homologous recombination by using the plasmid for preparing promoter varied strain constructed in the above-described step (6).

First, GC25 was transformed with plasmid pSFKTPDHApro3510 for preparing promoter modified strain by electrical pulse method (refer to J. P. KOKAI No. 2-207791). The cells were spread on CM2B mediu plates (comprising 10 g/l of polypeptone, 10 g/l of bactoyeast extract, 5 g/l of NaCl, 10 μ g/ml of biotin and 15 g/l of agar, and having pH 7.2) and cultured at 25℃ to obtain transformed strains. These transformants were cultured in CM2B liquid medium in a test tube overnight and then spread on CM2B medium plates containing 25 μ g /ml of kanamycin and cultured at 34℃ to obtain a a strain caused by once-recombination which contains plasmid

pSFKTPDHpro3510 on its chromosome inserted by the homologous recombination. After single colony isolation, this strain was cultured in CM2B liquid medium in a test tube overnight. After the suitable dilution, it was spread on CM2B medium plates and cultured at 31.5℃. After the colonies began to appear, the repllicas were made on CM2B medium plates containing 25 μ g/ml of kanamycin to obtain kanamycin-sensitive strains. Since two kinds of the strains, i.e. a strain having the sequence of wild type strain for the promoter region of pdhA gene and another strain having the mutation introduced therein, could be occured, this region was sequenced. Thus, a promoter modified strain, wherein the mutation had been introduced into the promoter region of pdhA gene, was obtained. In this strain, -35 region and –10 region of promoter of pdhA gene had been changed to the consensus sequence of coryneform bacteria. This strain was named GD3510.

Strains wherein -35 region or –10 region of promoter of pdhA gene had been changed to the consensus sequence of coryneform bacteria were obtained in the same manner as that described above except that above described plasmid pSFKTPDHApro3510 for producing the promoter modified strain was replaced with plasmid pSFKTPDHApro35 and pSFKTPDHApro10 for producing promoter modified strains and they were named GD35 and GD10, respectively.

(8) Evaluation of the results of flask culture of pdhA gene promoter modified strains:

The flask culture for producing L-glutamic acid was conducted with three kinds of pdhA gene promoter modified strains obtained as described above. Each of the cells of the promoter modified strains GD3510, GD35, GD10 and GC25 obtained by the culture on CM2B medium plates was inoculated into a medium (comprising 1 liter of pure water containing 30 g of glucose, 1 g of KH_2PO_4, 0.4 g of $MgSO_4$ $7H_2O$, 30 g of $(NH_4)_2SO_4$, 0.01 g of $FeSO_4$ $7H_2O$, 0.01 g of $MnSO_4$ $7H_2O$, 15 ml of soybean hydrolyzate, 200 μ g of thiamine hydrochloride, 60 μ g of biotin and 50 g of $CaCO_3$; and having a pH adjusted to 8.0 with KOH). Then the culture was shaked at 31.5℃ until the sugar in the medium had been consumed. The obtained products were

inoculated into the medium of the same composition as that described above in an amount of 5%, and the shaking culture was conducted at 37℃ until sugar in the medium had been consumed. After the completion of the culture, the amount of L-glutamic acid accumulated in the culture liquid was determined with Biotic Analyzer AS-210 (a product of Asahi Chemical Industry Co., Ltd.). The results are shown in Table 19.

Table 19

Strain	L-glutamic acid (g/dl)
GC25	1.9
GD35	2.0
GD10	2.0
GD3510	2.1

It was apparent from the results that the obtained promoter modified strains provided improved Glu yields.

Example 6 Introduction of mutation into promoter region of arginosuccinate synthase gene:

1) Determination of DNA sequence in the upstream of argG gene:

In order to amplify argG gene of Brevibacterium flavum by PCR, the DNA sequences in the upstream and downstream regions of the ORF were determined. The determination of the DNA sequences was conducted by synthesizing a primer based on the known DNA sequence (Gen Bank accession AF030520) of ORF of argG gene of Corynebacterium glutamicom and using in vitro LA PCR cloning kit (Takara shuzo Co., Ltd.) in accordance with the instruction manual included in the kit. As primers, they were specifically used oligonucleotide (primers 1 and 2) having the DNA

sequences set out as Seq ID No. 42 and Seq ID No.43 for the upstream region, and oligonucleotide (primers 3 and 4) having the DNA sequences set out as Seq ID No.44 and Seq ID No.45 for the downstream region. The DNA sequences in the upstream and downstream region of argG were determined by completely digesting chromosome DNA of 2247 strain (ATCC14067), i.e., wild type strain of Brevibacterium flavum, with a restriction enzyme EcoRI, conducting first PCR with the primer 2 or 3 (having sequence No.43 or 44), and conducting second PCR with the primer 1 or 2 (having sequence No. 42 or 45).

2) Prediction of promoter region:

A promoter-like sequence in the upstream of ORF of argG gene was search for the above-described sequences with a commercially available software (GENETYX). The mutation was introduced into a region of the highest score (about 120 bp upstream of the first ATG). Then, the promoter activity was measured.

3) Introduction of mutations into promoter sequence, and determination of activity of mutant promoters:

Mutation-introducing primers 9, 10, 11, 12 or 13 and 7 (having sequence No. 50, 51, 52, 53, 54 or 48,respectively) for a region of the highest score were used, and the first PCR was conducted with chromosomal DNA of AJ12092 strain as a template. The second PCR was conducted with the same chromosomal DNA as the template by using the PCR product as the primer for 3'-end and also using the primer 8 having sequence No. 49 as the primer on 5'-end to obtain DNA fragments having the mutation introduced in the intended promoter region. To determine the activity of the mutant promoters, these DNA fragments were inserted into SmaI site of promoter probe vector pNEOL so that they were in the same direction with lacZ reporter gene to obtain plasmids pNEOL-1, pNEOL-2, pNEOL-3, pNEOL-4 and pNEOL-7. As a control for the activity, plasmid pNEOL-0 was constructed by inserting the DNA fragment, obtained by PCR using chromosomal DNA of AJ12092 strain and primers 7

and 8, into the upstream of lacZ gene of pNEOL.

pNEOL-0, pNEOL-1, pNEOL-2, pNEOL-3, pNEOL-4 and pNEOL-7 were introduced into AJ12092 strain,respectivly. The plasmids were introduced by electrical pulse method (J. P. KOKAI No. 2-207791). The transformants were selected on CM2G medium plates(comprising 1 liter of pure water containing 10 g of polypeptone, 10 g of yeast extract, 5 g of glucose, 5 g of NaCl and 15 g of agar, and having pH 7.2) containing 4 μ g/ml of chloramphenicol, as chloramphenicol-resistant strains.

These strains were each spread on an agar medium (containing 0.5 g/dl of glucose, 1 g/dl of polypeptone, 1 g/dl of yeast extract, 0.5 g/dl of NaCl and 5 μ g/l of chloramphenicol), and cultured at 31.5℃ for 20 hours. One aze of the cells thus obtained was inoculated into a medium [containing 3 g/dl of glucose, 1.5 g/dl of ammonium sulfate, 0.1 g/dl of KH_2PO_4, 0.04 g/dl of $MgSO_4$, 0.001 g/dl of $FeSO_4$, 0.01 g/dl of $MnSO_4$, 5 μ g/dl of VB_1, 5 μ g/dl of biotin and 45 mg/dl (in terms of N) of soybean hydrolyzate]. After the culture at 31.5℃ for 18 hours, β -galactosidase activity of the obtained cells was determined as described in Example 4(4).

Since β -galactosidase activity was detected in AJ12092/pNEOL-0 as shown in Table 20, it was found that the DNA fragment inserted into the upstream of the gene of lacZ structure functioned as a promoter. In addition, β -galactosidase activity of each of the plasmid-introduced strains was higher than that of AJ12092/pNEOL-0. It was thus found that the transcription activity was increased by the introduction of the mutation into the promoter-like sequence, as shown in Table 20.

Table 20

	Relative activity (AJ12092/pNEOL-0=1)
AJI12092	nd
AJI12092/pNEOL-0	1.0
AJI12092/pNEOL-1	2.8
AJI12092/pNEOL-2	2.7

AJI12092/pNEOL-3	1.8
AJI12092/pNEOL-4	0.8
AJI12092/pNEOL-7	3.0

4) Construction of a plasmid for introduction of mutation:

PCR was conducted by using primers 14 and 15 (having the sequence of Seq ID No. 55 and Seq ID No.56) with chromosomal DNA of AJ12092 strain as the template. These DNA fragments thus obtained were inserted into a smaI site in a multicloning site of cloning vector pHSG398 (a product of TaKaRa) to construct plasmid p0. Then, p0 was digested with restriction enzymes EcoRV and BspHI, and also pNEOL-3 and pNEOL-7 were digested with restriction enzymes EcoRV and BspHI. DNA fragments thus obtained were ligated to obtain mutation-introducing plasmids p3 (mutant derived from mutation-introducing primer 11) and p7 (mutant derived from mutation-introducing primer 13).

5) Introduction of mutation-introducing plasmids into Arg-producing bacterium:

Each of the plasmids thus obtained was introduced into Arg-producing bacterium of the strain Brevibacterium lactofermentum AJ12092 by electrical pulse method (J. P, KOKAI No. 2-207791). Since these plasmids could not autonomously replicate in Brevibacterium, only the strains obtained by integrating these plasmids into the chromosome by homologous recombination could be selected as Cm-resistant strains. Strains in which the mutation-introducing plasmid was integrated into the chromosome were selected as chloramphenicol-resistant strains on CM2G medium plates (comprising 1 liter of pure water containing 10 g of polypeptone, 10 g of yeast extract, 5 g of glucose, 5 g of NaCl and 15 g of agar, and having pH 7.2) containing 5 μg/ml of chloramphenicol. Then, Cm-sensitive strains were selected in which the promoter region of argG gene was replaced with the intended modified sequence.

As a result, a strain substituted with P3 sequence (AJ12092-P3) and a strain substituted with P7 sequence (AJ12092-P7) were obtained.

(6) Cloning of argG gene

Based on the DNA sequence determined as in (1), oligonucleotides (primers 5 and 6) having the DNA sequence set out in Seq ID No. 46 and Seq ID No.47 were synthesized to conduct PCR using chromosomal DNA of Brevibacterium flavum as a template. The PCR reaction was conducted in 25 cycles, each cycle consisting of 94℃ for 30 seconds, 55℃ for one second and 72℃ for 2 minutes and 30 seconds. The thus-obtained DNA fragment was cloned to SmaI site in multi-cloning site of cloning vector pSTV29 (Takara shuzo Co. Ltd.) toobtain pSTVargG. Furthermore, pargG was prepared by inserting into SalI site of pSTVargG a fragment containing the replication origin obtained by treating pSAK4 set out in Example 1 with SalI.

7) Introduction of pargG into Brev.:

pargG was introduced into the strain Brevibacterium lactofernentum AJ12092. Plasmid was introduced by electrical pulse method (J. P, KOKAI No. 2-207791). The transformant was selected as chloramphenicol-resistant strain on CM2G medium plates(comprising 1 liter of pure water containing 10 g of polypeptone, 10 g of yeast extract, 5 g of glucose, 5 g of NaCl and 15 g of agar, and having pH 7.2) containing 4 μ g/ml of chloramphenicol.

8) ArgG activity of promoter modified strains:

ArgG activities of the above-described two kinds of argG promoter modified strains and a strain obtained by amplifying argG with plasmid (AJ12092/pargG) were determined. These strains were each spread on a agar medium (containing 0.5 g/dl of glucose, 1 g/dl of polypeptone, 1 g/dl of yeast extract, 0.5 g/dl of NaCl and 5 μ g/l of chloramphenicol), and cultured at 31.5℃ for 20 hours. One aze of the cells thus obtained were inoculated into a medium [containing 3 g/dl of glucose, 1.5 g/dl of ammonium sulfate, 0.1 g/dl of KH_2PO_4, 0.04 g/dl of $MgSO_4$, 0.001 g/dl of $FeSO_4$, 0.01 g/dl of $MnSO_4$, 5 μ g/dl of VB_1, 5 μ g/dl of biotin and 45 mg/dl (in terms of N) of soybean hydrolyzate]. After the culture at 31.5℃ for 18 hours, ArgG activity of the

obtained cells was determined by the method described above [Journal of General Microbiology (1990), 136, 1177-1183]. ArgG activities of the above-described two kinds of ArgG promoter modified strains and the strain (AJ12092/pargG) obtained by amplifying argG with plasmid are shown in Table 21. It is apparent from Table 21 that by introducing the mutation into the promoter, ArgG activity of AJ12092-P3 was increased to about twice as high as that of the parent strain, and the activity of AJ12092-P7 was increased to about three times as high as that of the parent strain. ArgG activity of AJ12092/pargG was about 4.5 times as high as that of the parent strain.

Table 21

	Relative activity (AJ12092=1)
AJI12092	1.0
AJI12092-P3	2.1
AJI12092-P7	2.9
AJI12092/pargG	4.4

9) Arg production by promoter modified strains:

The flask culture of each of argG promoter modified strains was conducted. As controls, parent strain AJ12092 and AJ12092/pargG were also cultured. These strains were each inoculated into a medium [containing 0.1 g/dl of KH_2PO_4, 0.04 g/dl of $MgSO_4$, 0.001 g/dl of $FeSO_4$, 0.01 g/dl of $MnSO_4$, 5 μ g/dl of VB_1, 5 μ g/dl of biotin and 45 mg/dl (in terms of N) of soybean hydrolyzate]; and then spread on an agar medium (containing 0.5 g/dl of glucose, 1 g/dl of polypeptone, 1 g/dl of yeast extract, 0.5 g/dl of NaCl and 5 μ g/l of chloramphenicol), and cultured at 31.5℃ for 20 hours. One aze of the cells were cultured in a flask containing 4 g/dl of glucose and 6.5 g/dl of ammonium sulfate at 31.5℃ until glucose had been completely consumed. The absorbance (CD620) of the culture liquid diluted to a concentration of 1/51 with 0.2 N HCl solution, the quantity of arginine produced (concentration: g/dl) and culture time

were shown in Table 22.

It is apparent from Table 22 that when argG promoter modified strain was used, the yield of arg was increased to a level equal to that of argG amplified with plasmid. As for the promoter varied strains, both AJ12092-P3 and AJ12092-P7 had the culture time equal to that of the parent strain, while the culture time of the plasmid amplified strain was increased. It was thus apparent that Arg productivity thereof was higher than that of the plasmid amplified strain.

Table 22

	OD	Arg (g/dl)	Culture time (h)	Productivity (g/dl/h)
AJI2092	0.502	1.25	48	0.026
AJI2092-P3	0.510	1.47	48	0.031
AJI2092-P7	0.514	1.43	48	0.030
AJI2092/pargG	0.520	1.47	52	0.028

Example 7 Introduction of mutation into GDH gene promoter region of coryneform glutamate-producing bacterium:

(1) Construction of mutant gdh plasmids:

Plasmids having GDH promoter sequence of FGR1 strain and FGR2 strain described in Example 2 were constructed by site directed mutagenesis. For obtaining GDH promoter sequence of FGR1 strain, PCR was conducted by using synthetic DNA shown in Seq ID No. 57 and synthetic DNA shown in No. 60 as the primers and chromosomal DNA of ATCC13869 as the template; and on the other hand, PCR was conducted by using synthetic DNA shown in Seq ID No. 58 and synthetic DNA shown in Seq ID No. 59 as the primers with chromosomal DNA of ATCC13869 as the template. Further, PCR was conducted by using synthetic DNAs shown in Seq ID Nos. 57 and Seq ID No.58 as the primers with a mixture of these

PCR products as the template. The PCR product thus obtained was inserted into SmaI site of pSFKT2 (Japanese Patent Application No. 11-69896) to construct pSFKTG11. To obtain GDH promoter sequence of FGR2 strain, PCR was conducted by using synthetic DNA shown in Seq ID No. 57 and synthetic DNA shown in Seq ID No. 62 as the primers and chromosomal DNA of ATCC13869 as the template; and on the other hand, PCR was conducted by using synthetic DNA shown in Seq ID No. 58 and synthetic DNA shown in Seq ID No. 61 as the primers and chromosomal DNA of ATCC13869 as the template. Further, PCR was conducted by using synthetic DNA shown in Seq ID No. 57 and Seq ID No.58 as the primers and a mixture of these PCR products as the template. The PCR product thus obtained was inserted into SmaI site of pSFKT2 (Japanese Patent Application No. 11-69896) to construct pSFKTG07. The DNA sequences of the fragments inserted into SmaI sites of pSFKTG11 and pSFKTG07 were determined to confirm that no mutation was introduced into other refions than the promoter region in GDH.

(2) Construction of gdh promoter modified strains:

Then, pSFKTG11 and pSFKTG07 were introduced into AJ13029 strain by electrical pulse method, and transformants which grew on CM2B plates containing 25 μg/ml of kanamycin at 25℃ were selected. The transformants were cultured at 34℃ to select strains which were resistant to kanamycin at 34℃. The fact that a strain is resistant to kanamycin at 34℃ indicates that pSFKTG11 or pSFKTG07 was thus integrated on the chromosome of AJ13029 strain. Kanamycin-sensitive strains were obtained from the strains in which the plasmid was integrated on the chromosome. The GDH promoter sequences of these strains were determined. The strains having the same gdh promoter sequence as those of pSFKTG11 and pSFKTG07 were named GA01 and GA02, respectively.

(3) Confirmation of L-glutamic acid-productivity of gdh promoter modified strains:

The glutamic acid productivities of strains GA01 and GA02 and the parent strain

AJ13029 were confirmed in the same manner as that of Example 2 (2) given above. As a result, a remarkable improvement in the accumulation of glutamic acid was recognized in GA01 and GA02 as shown in Table 23.

Table 23

Strain	Glu (g/dl)	Specific activity of GDH	Relative value
AJ13029	2.6	7.7	1.0
GA01	3.0	22.3	2.9
GA02	2.9	27.0	3.5

(4) Construction of self-cloning type gdh plasmid:

First, self-cloning vector pAJ220 was constructed. pAJ226 (J. P. KOKAI No.61-152289) was treated with EcoRV and PstI to prepare a fragment containing a region which could be autonomously replicated in a coryneform bacterium. The fragment was ligated with about 0.7 kb of the DNA fragment obtained by treating pAJ224 (J. P. KOKAI No. Sho 61-152289) with EcoRV and PstI to obtain a plasmid pAJ220. This plasmid could autonomously replicate in a coryneform bacterium, and it could afford trimethoprim resistance to the host.

PCR reaction was conducted by using synthetic DNA shown in Seq ID No. 63 and Seq ID No.64 as the primers and chromosomal DNA of wild-type coryneform bacterium strain ATCC13869 as the template. The gdh gene fragment thus obtained was inserted in BalI site of pAJ220 to construct pAJ220G. The promoter was present near BalI site of pAJ220, and the expression of the inserted gene was increased depending on the direction of the gene inserted into BalI site. PAJ220G and pGDH were introduced into ATCC13869 strain by electrical pulse method. GDH activities of the strains thus constructed were determined by the method stated in above-described step (1). As a result, GDH activity of the strain into which pAJ220G had been introduced was about 1.5 times as high as that of the strain into which dGDH had been introduced as shown in Table 24.

Table 24

Strain	Specific activity of GDH	Relative value
ATCC13869	7.7	1.0
ATCC13869/pGDH	82.7	10.7
ATCC13869/pAJ220G	120.l	15.6

(5) Investigations on influence of gdh activity on the yield and by-produced Asp:

pGDH and pAJ220G were introduced into AJ13029 by electrical pulse method. Each of these strains and those obtained in above-described step (2) was inoculated into a seed culture medium having a composition shown in Table 25, and the shaking culture was conducted at 31.5℃ for 24 hours to obtain the seed culture. 300 ml of medium for main culture having a composition shown in Table 25 was placed in each of 500 ml glass jar farmenters and then sterilized by heating. 40 ml of the seed cultures as described above were inoculated into the medium. The culture was started at a temperature of 31.5℃ while the stirring rate and the aeration rate were controlled at 800 to 1300 rpm and 1/2 to 1/1 vvm, respectively. The pH of the culture liquid was kept at 7.5 with gaseous ammonia. The temperature was shifted to 37℃ 8 hours after the initiation of the culture. The culture was terminated when glucose had been completely consumed in 20 to 40 hours, and the quantity of L-glutamic acid produced and accumulated in the culture liquid were determined (Table 26). The GDH activity for obtaining the highest yield was about 3-times as high. When GDH activity was further elevated, the degree of the improvement in the yield was reduced. When the GDH activity was elevated to about 16-times, the yield was rather reduced. Amino acids produced as by-products were analyzed with Hitachi Amino Acid Analyzer L-8500 to find that as GDH activity was elevated, the amount of accumulated aspartic acid and alanine was increased. These results proved the following facts: For increasing the yield of glutamic acid, it is necessary to suitably increase GDH activity so as not to cause a remarkable increase in the amount of aspartic acid and

alanine. One of the effective methods therefor comprises the introduction of various mutations into gdh promoter to control GDH activity to about 3-times as high as that of the parent strain.

Table 25

	Concentration	
Ingredient	Seed culture	Main culture
Glucose	50 g/l	150 g/l
KH_2PO_4	1 g/l	2 g/l
$MgSO_4 \cdot 7H_2O$	0.4 g/l	1.5 g/l
$FeSO_4 \cdot 7H_2O$	10 mg/l	15 mg/l
$MnSO_4 \cdot 4H_2O$	10 mg/l	15 mg/l
Soybean protein hydrolyzate	20 ml/l	50 ml/l
Biotin	0.5 mg/l	2 mg/l
Thiamine hydrochloride	2 mg/l	3 mg/l

Table 26

Strain	Glu (g/dl)	Asp (mg/dl)	Ala (mg/dl)	Relative activity of GDH	Relative value
AJ 13029	8.3	49	60	7.7	1.0
GA01	9.0	145	152	22.3	2.9
GA02	8.9	153	155	27.0	3.5
AJ13029/pGDH	8.8	201	190	82.7	10.7
AJ13029/pAJ220G	7.5	290	590	120.12	15.6

Sequence Listing

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Tyr Val Asn Thr Ile Pro Thr Ser Met Glu Pro Glu Phe Pro Gly Asp
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Asn Leu Asp Asn Leu Thr Phe Val Val Asn Cys Asn Leu Gln Arg Leu
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What is claimed is

1. A method of producing coryneform bacteria having an improved amino acid- or nucleic acid-productivity, which comprises the steps of introducing a mutation in a promoter sequence of amino acid- or nucleic acid-biosynthesizing genes on a chromosome of a coryneform bacterium to make it close to a consensus sequence or introducing a change in a promoter sequence of amino acid- or nucleic acid-biosynthesizing genes on a chromosome of a coryneform bacterium by gene recombination to make it close to a consensus sequence, to obtain a mutant of the coryneform amino acid- or nucleic acid-producing microorganism, culturing the mutant and selecting a mutant capable of producing the intended amino acid or nucleic acid in a large amount.

2. The method of claim 1, wherein the amino acid is selected from the group consisting of glutamic acid, lysine, arginine, serine, phenylalanine, proline and glutamine, and nucleic acid is selected from the group consisting of inosine, guanosine, adenosine and nucleotide.

3. The method of claim 1, wherein the amino acid is glutamic acid, and the promoter for the biosynthesizing gene is selected from the group consisting of a promoter for glutamate dehydrogenase (GDH) gnene, a promoter for citrate synthase (CS) gene, a promoter for isocitrate synthase (ICDH) gnene, a promoter for pyruvate dehydrogenase (PDH) gene and a promoter for aconitase (ACO)-producing gene.

4. The method of claim 3, wherein the promoter for glutamate dehydrogenase (GDH) gene has a DNA sequence selected from the group consisting of (i) at least one DNA sequence selected from the group consisting of CGGTCA, TTGTCA, TTGACA and TTGCCA in -35 region (ii) TATAAT sequence or the same TATAAT sequence but in which the base of ATAAT is replaced with another base in -10 region, and (iii) a combination of (i) and (ii), wherein the sequence does not inhibit the function of the promoter.

5. The method of claim 4, wherein the promoter for GDH has TGGTCA in –35 region,

and TATAAT in –10 region; or TTGTCA in –35 region, and TATAAT in –10 region.

6. The method of claim 3, wherein the promoter for CS has (i) TTGACA sequence in –35 region, (ii) TATAAT sequence in –10 region, or (iii) a sequence of the combination of (i) and (ii), and the sequence does not inhibit the function of the promoter.

7. The method of claim 3, wherein at least one of first and second promoters for ICDH has (i) TTGCCA or TTGACA sequence in –35 region, (ii) TATAAT sequence in –10 region, or (iii) a sequence of the combination of (i) and (ii), and the sequence does not inhibit the function of the promoter.

8. The method of claim 3, wherein the promoter for PDH has (i) TTGCCA sequence in –35 region, (ii) TATAAT sequence in –10 region, or (iii) a sequence of the combination of (i) and (ii), and the sequence does not inhibit the function of the promoter.

9. The method of claim 1, wherein the amino acid is arginine, and the promoter for the biosynthesizing gene is a promoter for argininosuccinate synthase.

10. The method of claim 9, wherein the promoter for the argininosuccinate synthase has a DNA sequence selected from the group consisting of (i) at least one DNA sequence selected from the group consisting of TTGCCA, TTGCTA and TTGTCA in -35 region, (ii) TATAAT sequence or TATAAT sequence but in which the base of ATAAC is replaced with another base in -10 region, and (iii) a combination of (i) and (ii) and the sequence does not inhibit the promoter function.

11. The method of claim 10, wherein the promoter for the argininosuccinate synthase has a DNA sequence selected from the group consisting of (i) TTGTCA in -35 region, (ii) TATAAT sequence in -10 region, and (iii) a combination of (i) and (ii).

12. A glutamic acid-synthesizing gene having a promoter according to any one of claims 4 to 8.

13. An arginine synthetase gene having the promoter of claim 10.

14. A coryneform glutamic acid-producing bacterium having the glutamate synthetase gene of claim 12.

15. A coryneform arginine-producing bacterium having the arginine synthetase gene

of claim 13.

16. A method of producing an amino acid or nucleic acid by the fermentation, which comprises the steps of culturing a coryneform bacterium constructed by the method of any one claims 1 to 11 and having an improved amino acid- or nucleic acid-productivity, or the coryneform bacterium of claims 14 or 15 in a culture medium to form and thereby to accumulate the intended amino acid or nucleic acid in the culture medium, and collecting it from the culture medium.

17. A method of producing L-glutamic acid by fermentation, which comprises the steps of culturing a coryneform L-glutamic acid-producing bacterium resistant to 4-fluoroglutamic acid in a liquid culture medium to produce and thereby to accumulate L-glutamic acid in the culture medium, and collecting it from the culture medium.

Abstract of the disclosure

A method of producing coryneform bacteria having an improved amino acid- or nucleic acid-productivity comprises the steps of introducing a mutation in a promoter sequence of amino acid- or nucleic acid-biosynthesizing genes on a chromosome of a coryneform bacterium to make it close to a consensus sequence or introducing a change in a promoter sequence of amino acid- or nucleic acid-biosynthesizing genes on a chromosome of a coryneform bacterium by gene recombination to make it close to a consensus sequence, to obtain mutants of the coryneform amino acid- or nucleic acid-producing microorganism, culturing the mutants and select a mutant capable of producing the intended amino acid or nucleic acid in a large amount. This method can construct a mutant capable of suitably enriching or controling the expression of an intended gene without using a plasmid and also capable of producing amino acids in a high yield, by the recombination or mutation.

FIG. 1

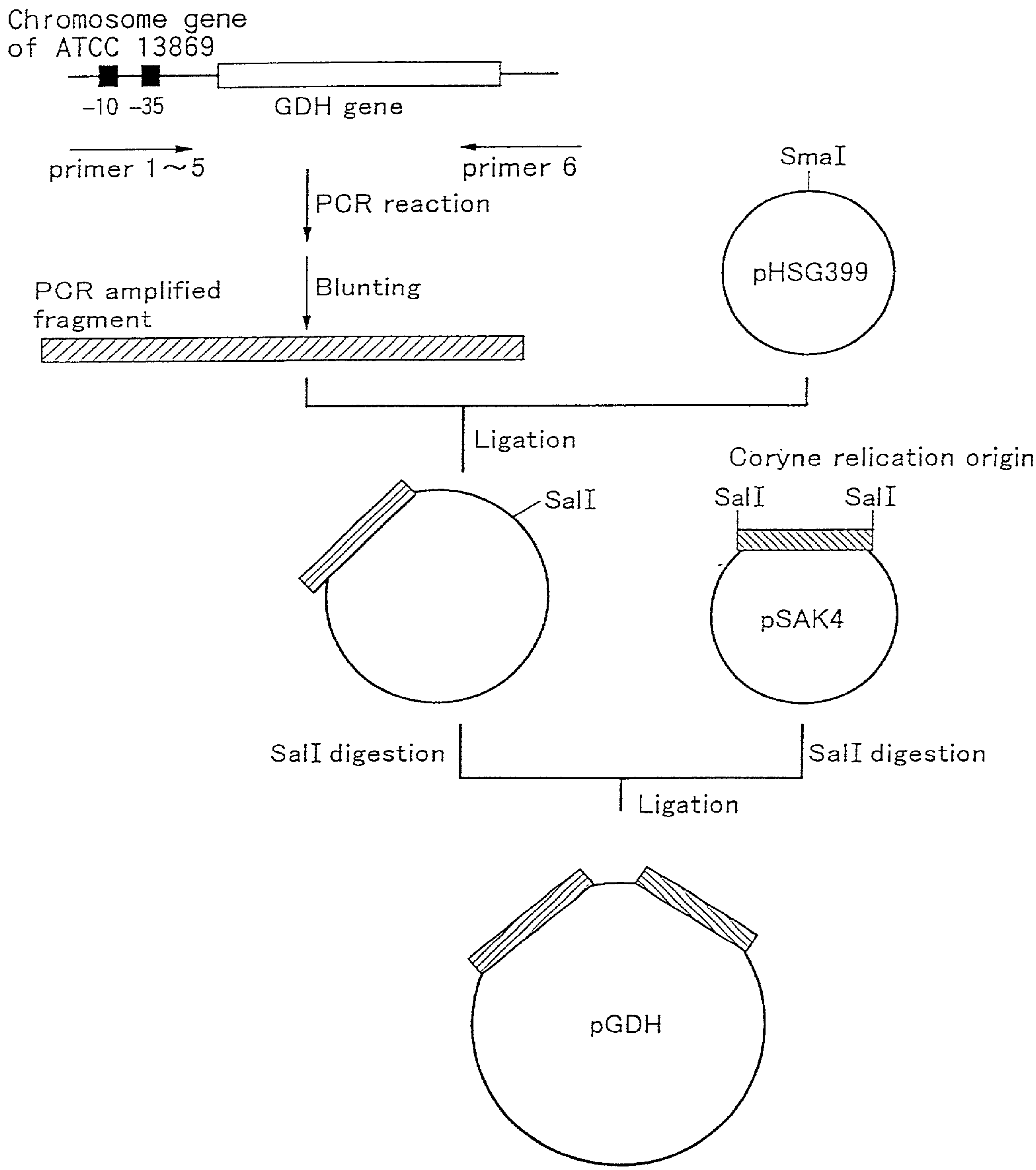
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FIG. 2

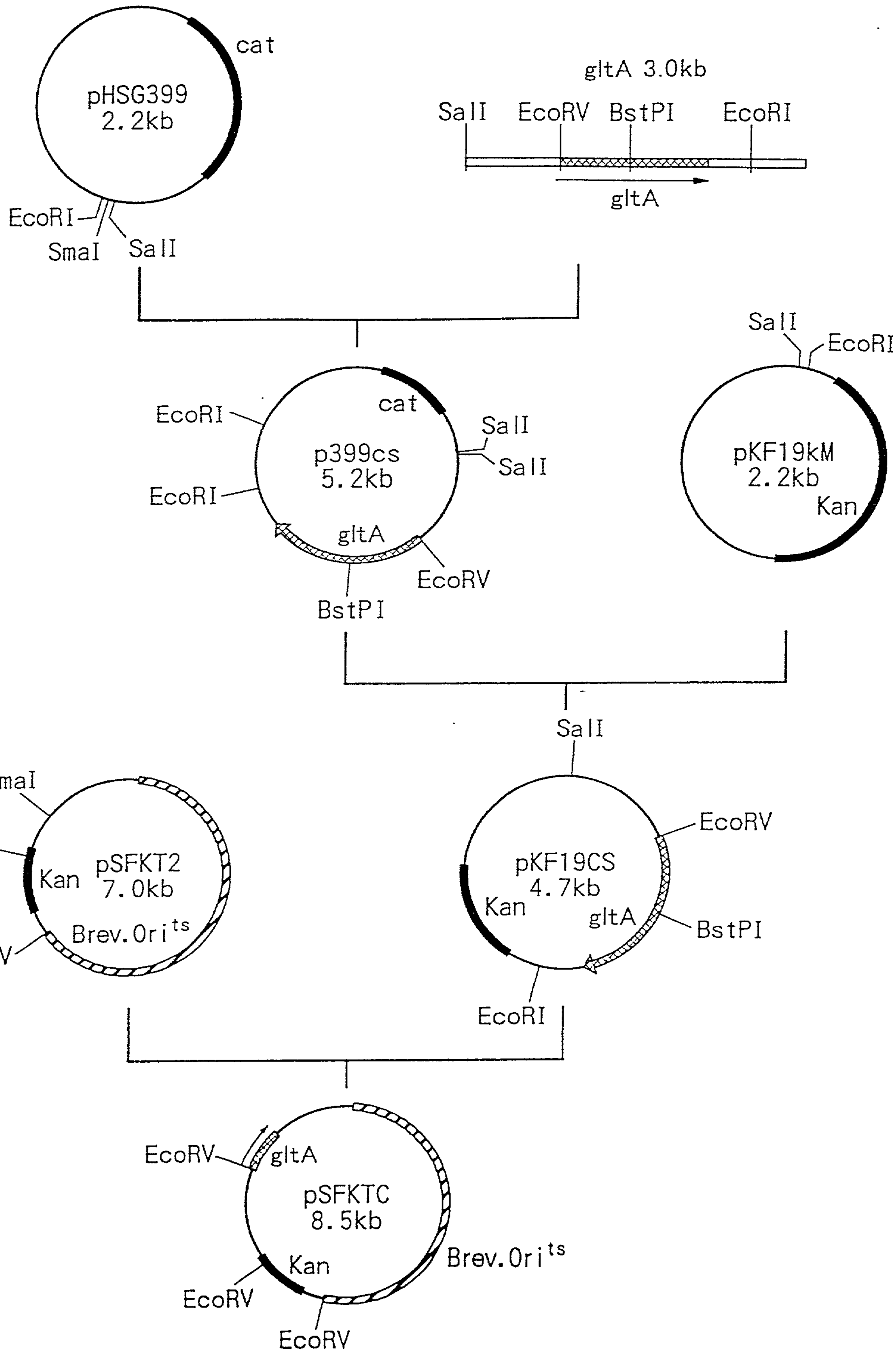
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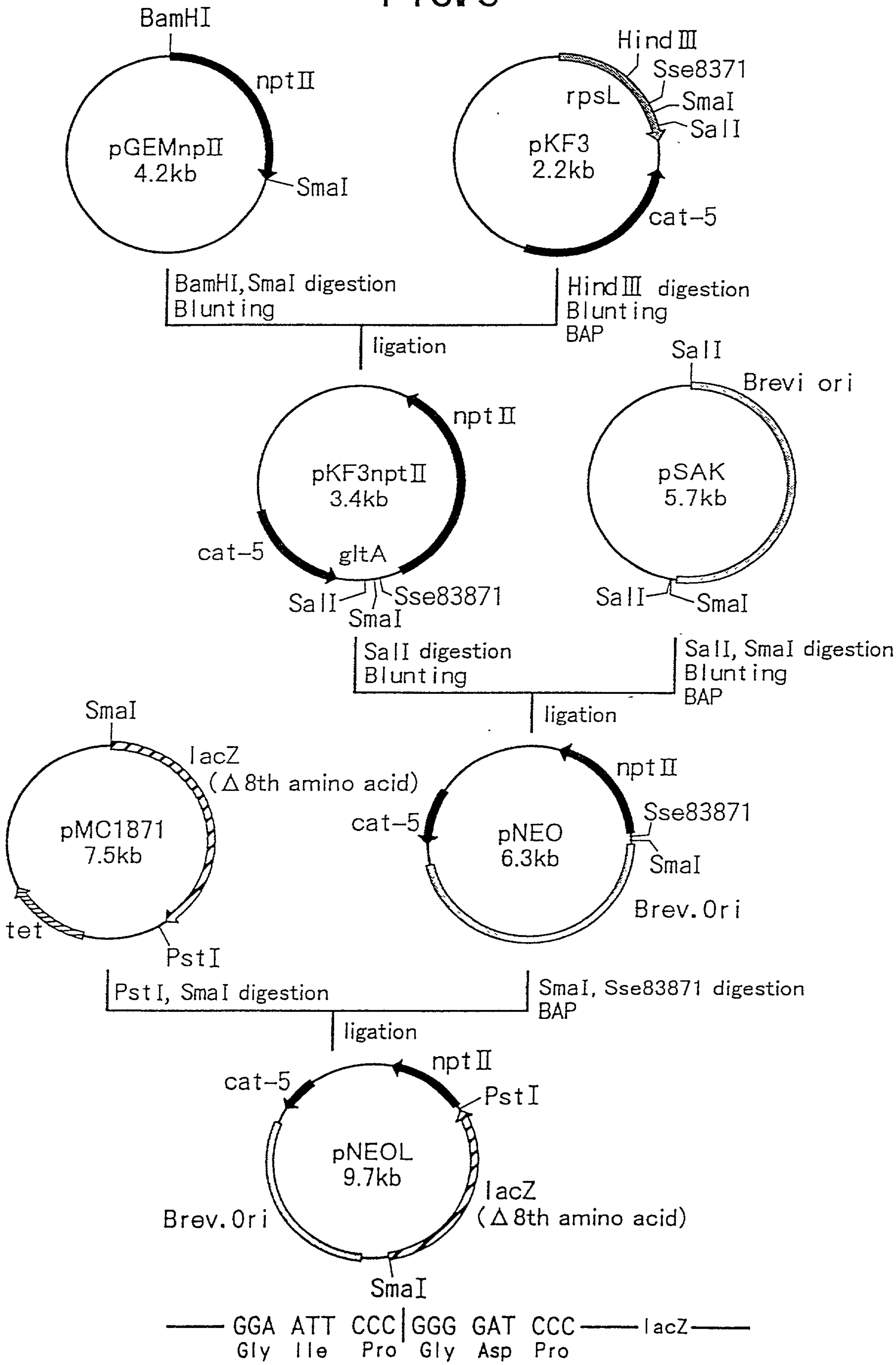
FIG. 3



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Gly Ile Pro Gly Asp Pro

OP99052-6

Declaration and Power of Attorney For Patent Application

特許出願宣言書及び委任状

Japanese Language Declaration

日本語宣言書

下記の氏名の発明者として、私は以下の通り宣言します。

As a below named inventor, I hereby declare that:

私の住所、私書箱、国籍は下記の私の氏名の後に記載された通りです。

My residence, post office address and citizenship are as stated next to my name.

下記の名称の発明に関して請求範囲に記載され、特許出願している発明内容について、私が最初かつ唯一の発明者（下記の氏名が一つの場合）もしくは最初かつ共同発明者（下記の名称が複数の場合）であると信じています。

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled.

Method of constructing amino acid producing bacterial strains, and method of preparing amino acids by fermentation with the constructed amino acid producing bacterial strains

上記発明の明細書は、

☐ 本書に添付されています。

☐ ____月____日に提出され、米国出願番号または特許協定条約国際出願番号を____________とし、（該当する場合）____________に訂正されました。

the specification of which

☒ is attached hereto.

☐ was filed on ____________________ as United States Application Number or PCT International Application Number ____________ and was amended on ____________ (if applicable).

私は、特許請求範囲を含む上記訂正後の明細書を検討し、内容を理解していることをここに表明します。

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

私は、連邦規則法典第37編第1条56項に定義されるとおり、特許資格の有無について重要な情報を開示する義務があることを認めます。

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

Japanese Language Declaration
(日本語宣言書)

私は、米国法典第35編119条（a）-（d）項又は365条（b）項に基づき下記の、米国以外の国の少なくとも一ヵ国を指定している特許協力条約365（a）項に基づく国際出願、又は外国での特許出願もしくは発明者証の出願についての外国優先権をここに主張するとともに、優先権を主張している、本出願の前に出願された特許または発明者証の外国出願を以下に、枠内をマークすることで、示しています。

I hereby claim foreign priority under Title 35, United States Code, Section 119 (a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)
外国での先行出願

(Number) (番号)	(Country) (国名)	(Day/Month/Year Filed) (出願年月日)	Priority Claimed 優先権主張 Yes はい	No いいえ
271786/1998	Japan	25/09/1998	☒	☐
271787/1998	Japan	25/09/1998	☒	☐

私は、第35編米国法典119条（e）項に基づいて下記の米国特許出願規定に記載された権利をここに主張いたします。

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below.

(Application No.) (出願番号)	(Filing Date) (出願日)

私は、下記の米国法典第35編120条に基づいて下記の米国特許出願に記載された権利、又は米国を指定している特許協力条約365条（c）に基づく権利をここに主張します。また、本出願の各請求範囲の内容が米国法典第35編112条第1項又は特許協力条約で規定された方法で先行する米国特許出願に開示されていない限り、その先行米国出願書提出日以降で本出願書の日本国内または特許協力条約国際提出日までの期間中に入手された、連邦規則法典第37編1条56項で定義された特許資格の有無に関する重要な情報について開示義務があることを認識しています。

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of application.

(Application No.) (出願番号)	(Filing Date) (出願日)	(Status: Patented, Pending, Abandoned) (現況：特許許可済、係属中、放棄済)
PCT/JP99/05175	September 22, 1999	Pending

私は、私自信の知識に基づいて本宣言書中で私が行なう表明が真実であり、かつ私の入手した情報と私の信じるところに基づく表明が全て真実であると信じていること、さらに故意になされた虚偽の表明及びそれと同等の行為は米国法典第18編第1001条に基づき、罰金または拘禁、もしくはその両方により処罰されること、そしてそのような故意による虚偽の声明を行なえば、出願した、又は既に許可された特許の有効性が失われることを認識し、よってここに上記のごとく宣誓を致します。

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Japanese Language Declaration
(日本語宣言書)

委任状：私は下記の発明者として、本出願に関する一切の手続きを米特許商標局に対して遂行する弁理士または代理人として、下記の者を指名いたします。
(弁護士、または代理人の指名及び登録番号を明記のこと)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: *(list name and registration number)*

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(第三以降の共同発明者についても同様に記載し、署名すること)

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Japanese Language Declaration
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Japanese Language Declaration
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第十二の共同発明者の氏名	Full name of twelfth joint inventor, if any
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